BINDING CHARACTERISTICS AND ENZYME INHIBITION PROPERTIES OF CONSTITUENTS OF COMMERCIAL ORGANOPHOSPHORUS PESTICIDES

Thesis

SUBMITTED TO THE PUNJAB AGRICULTURAL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE
IN
BIOCHEMISTRY

(Minor Subject : Microbiology)

By

REENA THAPAR
(L-96-BS-142-M)

Department of Biochemistry
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LUDHIANA-141 004

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CERTIFICATE I

This is to certify that the thesis entitled, **"Binding characteristics and enzyme inhibition properties of constituents of commercial organophosphorus pesticides"** submitted for the degree of M.Sc., in the subject of **Biochemistry** (Minor subject: **Microbiology**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Reena Thapar(L-96-BS-142-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

6/5/99

Major Advisor
(Dr.S.P.Ahuja)
Professor of Biochemistry
National Fellow of ICAR(85-88)
Department of Biochemistry
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Ludhiana-141004

CERTIFICATE II

This is to certify that the thesis entitled, **"Binding characteristics and enzyme inhibition properties of constituents of commercial organophosphorus pesticides"** submitted by **Reena Thapar (L-96-BS-142-M)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of M.Sc., in the subject of Biochemistry (Minor subject: Microbiology) has been approved by the Students' Advisory Committee after an oral examination on the same in collaboration with an External Examiner.

23/7/99
for Dr S.P Arora
Major Advisor

23.07.99
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ABSTRACT

TLC of Ethion, Phorate, Phenthoate, Malathion, Methyl-parathion, Quinalphos, Dimethoate and Monocrotophos showed 4 to 8 phosphorus containing components. The lower detection limits of major components of Quinalphos, Methylparathion and Dimethoate were 624 ppb, 938 ppb and 521 ppb, respectively with iodine vapour and Lowry reagents. On reaction with Lowry reagents Dimethoate, Monocrotophos and Malathion; Methyl parathion; Quinalphos; and Ethion, Phenthoate and Phorate could be estimated at 660 nm, 410 nm, 380 nm and 520/660 nm, respectively. The Lowry method for estimation of proteins was adopted for detection/estimation of organophosphorus pesticides and their residues. The binding of pesticides (50 μg to 250μg a.i.) to lipase, proteases, proteins and microsomes increased in a disproportionate manner, with increasing concentration of pesticides. Inhibition of tyrpsin and pepsin was 80% and 86.5% with 250 μg a.i. of Phorate and Phenthoate, respectively. The binding of the eight pesticides to microsomes from brain was higher than to those from liver, spleen, kidneys and testes. The Absolute Absorption Spectra of microsomal preparations from these tissues of goats showed hyperchromic effect with all the pesticides except for Phenthoate and Malathion with microsomes of liver. In general Absorption Difference Spectra showed that the binding of pesticides to microsomes from spleen, brain,

kidneys is at lipophillic site away from heme or directly to heme in microsomes from testes and liver. The microsomal esterase(s) from these tissues and from abomasal-, duodenal mucosal cells showed maximum activity at pH 7.4 and 8.5 (except brain pH 8.0), at 37°C (except 45°C for esterases from spleen). At least two esterases were present in abomasal-, duodenal mucosal cells and in microsomes from liver, kidneys, brain, spleen and testes. Microsomes from different tissues showed substrate inhibition at [S]>0.8 μM. The microsomal esterases from liver, kidney, spleen and brain showed concentration dependent inhibition with all the pesticides. From the binding and enzyme inhibition studies it appears that microsomes, proteins and hydrolytic enzymes sequester the pesticides and decrease their toxicity. The comparative structure and toxicity studies with these eight pesticides showed that phosphorothioates and phosphates with aliphatic and aromatic groups mainly affect the organisation and functions of microsomes from brain and kidneys. Dithiophosphates with thioether or carbamide linkage in the side chain affect testes also. The presence of aromatic group in phosphorothioates also produces such effects in spleen and/or testes.

6/5/99

Signature of Major Advisor

Reena Thapar

Signature of student

CONTENTS

CHAPTER 1

INTRODUCTION

The use of chemicals as fertilizers and pesticides in intensive agricultural practices have increased the yield of crops. India is the second largest manufacturer of basic pesticides (Anon., 1998). The average consumption of pesticides in India during 1989-90 was 450g per hectare. During the period 1954-89, the use of pesticides increased from 434 tonnes to 74,418 tonnes. Now 92,000 tonnes of pesticides per annum, are used in India, which forms 3.75% of world consumption of pesticides. This is likely to increase to 100,000 tonnes in the year 2000 and for agriculture alone. However, in India, the level of contamination of food chain with pesticides is higher than that in developed countries although their utilization of pesticides is larger. The reasons for this are evident from the report of Joia *et al.*(1998) who observed that cauliflower grown in Punjab are sprayed with recommended (and not recommended) pesticides at intervals of 2-15 days instead of the recommended interval of 10 days. Such blanket sprays on vegetables are common during the off season when prices are high. This is ascribed to poor awareness of the farmers and public in general about the consequences of excessive and indiscriminate use of pesticides. This lack of

concern for safety has introduced the pesticide(s) residues into the food chain and will create problems of slow toxicity after prolonged consumption of contaminated farm products of plant and animal origin.

Due to their long half life and residual effect the chlorinated hydrocarbon pesticides have been replaced by organophosphorus pesticides, for the control of pests of agricultural, veterinary and medical importance. Some of the organophosphorus pesticides are also known to have long half life. The information on the organophosphorus pesticide residues in agricultural products, soil, water and air is scanty and monitoring of such residues was recommended during a national level discussion on integrated pest management (Bakhetia, 1998). The effects and mechanism of action of acute toxicity due to organophosphorus pesticides is well documented. However, little is known about toxicity due to prolonged exposure to low doses of organophosphorus pesticides, through residues in the environment. The review of literature shows that the relationship between structure of organophosphorus pesticides and binding to biomolecules or effects on their functions too have been studied to a limited extent. Moreover, the contribution of dietary and digestive tract biomolecules in modifying the effects of commercially available organophosphorus pesticides has not been investigated. Such studies will be useful in understanding the biochemical basis of development of toxicity after prolonged exposure to pesticide residues in the environment. In 1969, Swaminathan had predicted that the indiscriminate use of pesticides, fungicides

and herbicides could cause adverse changes in ecological imbalance and may increase the incidence of cancer and other diseases through toxic residues present in the grains or other edible parts (Swaminathan, 1999). The farm animals and public in general, the workers in municipality health services, workers in pesticide and agriculture industries are likely to get exposed to variable doses of pesticides by dermal contact, inhalation and/or by eating the contaminated foods.

The levels of organophosphorus pesticide residues in foods, extent of their binding to biomolecules, adsorption/absorption and inhibition of various enzymes have been investigated to a limited extent, especially after prolonged exposure to low doses of pesticides. *In vitro* studies with goat tissues and commonly used organophosphorus pesticides were, therefore, carried out with the following objectives:

- To fractionate, characterize and estimate the constituents of commercial formulation of Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos.
- To explain the mechanism(s) of action of organophosphorus pesticides the following aspects were investigated:
  - Absorption spectra of pesticides, microsomes and their complexes
  - *In vitro* binding of pesticides to biomolecules
  - *In vitro* binding of digestive enzymes, microsomes, and microsomal esterase(s) by pesticides and kinetics, inhibition of these enzymes.

CHAPTER II

REVIEW OF LITERATURE

The pesticides form a variety of chemicals with different modes of metabolism and toxicity. In agriculture, pesticides are used for control of pests of plants, animals and man. Initially chlorinated hydrocarbon pesticides were used (Müller, 1939). Their use has been banned because of their accumulation/persistence in tissues, environment and higher toxicity to mammals (Anon., 1972). Moreover, the pests have developed resistance to these pesticides, by inducing detoxifying enzymes and/or mechanisms to modify their uptake. Therefore, the use of organophosphorus pesticides, which are selectively more toxic to pests than to mammals were introduced. The acute toxicity of organophosphorus pesticides is high and has been extensively studied and explained. However, little effort has been made to understand the effects and mechanism of their action after prolonged administration in very low doses. Anon.(1990) reported distribution of proportion of population groups at risk of pesticides (Fig.1). When two or more pesticides are used simultaneously, the toxicity may be antagonistic or synergistic e.g. Lindane with Heptachlor; interaction of dietary nitrite with pesticides may form more toxic, mutagenic nitrosamines. The highly toxic pesticides have LD_{50} of upto 50 mg per kg body weight and have

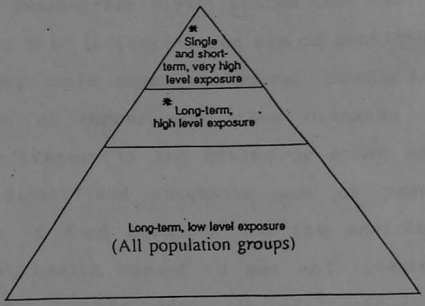


Fig. 1 Relative proportion of population groups at risk of exposure to pesticides. * Poisoning cases and workers in Public Health, Agricultural and Pesticide Industries. (Modified from Anon. 1990)

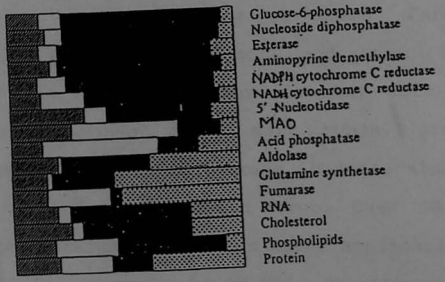


Fig.2 Partition of the enzymes, chemical constituents between nuclear (M), large granules (ML), microsomal (P) and supernatant (S) fractions N ▨ ML □ P ■ S ▩ % of amount recovered. (Modified from Amar-Costesec et al., 1969)

a half-life of more than one month in human tissues (Anon., 1990).

Swaminathan (1999) stated that earlier in 1969, he had predicted that indiscriminate use of pesticides, fungicides and herbicides could cause ecological imbalance and increase the incidence of cancer and other diseases through the toxic residues present in the grains or other edibles. Due to the indiscriminate and excessive use of pesticides, pesticide residues in food products and the environment constitute a potential health hazard to man and livestock. The available literature on the detection of residues and toxicity of organophosphorus pesticides has been reviewed.

2.1 Epidemiology and Detection of Pesticide Residues

Krishnaiah and Kalode (1992) reported persistence of granular insecticides (Carbofuran, Phorate and Aldicarb) in soils. Aldicarb was least persistent without any appreciable difference among the type of soils. Carbofuran persisted maximally in sandy loam soil and least in alkaline soil. Phorate was least persistent in clay soil and there was no difference between other soil types. Singh *et al.*(1991) reported persistence of Phorate in soil, along with its uptake and metabolism in groundnut crop. Over 90 per cent of the insecticide present at 7 days after application (DAA; 1 kg or 4 kg ha^{-1}) was degraded by 45 DAA. Phorate residue in groundnut was >1 ppm at 30 DAA but was not detectable by 90 DAA or 127 DAA. Gonazales-Lopez *et al.*(1993) reported that Phorate at 300 fg g^{-1} or Malathion at 100-300 fg g^{-1} significantly decreased dinitrogen fixation in soil microorganisms. The presence of

10-300 fg g^{-1} of Malathion significantly reduced the total number of soil bacteria but the number of denitrifying bacteria were increased by Malathion (50-300 fg g^{-1}). Nitrifying bacteria and fungi were unaffected by these insecticides. By using HPLC (230 nm UV detector) Hung *et al.*(1994) reported that the detection limits in soil were 2 pg for Dialifos,5 pg for Mephosfolan, Fensulfonthion, Chlorfenvinphos, Fonofos, Phoxim, Chlorpyrifos and Carbophenothion and 20 pg for Phorate, Disulfoton and Terbufos. Sharma and Sharma (1992) reported that Carbaryl and Aldicarb sulfone (Aldoxycarb) were highly nematicidal against *Paratylenchus prunii* at 10 ppm in water and at 20 ppm in soil after 2 days. Doraisamy and Rajukkannu (1990) reported that half life of Dichlorvos and Malathion in soils was 13 to 32 days, whereas Quinalphos and Methyl parathion persist longer than Malathion. Puccetti *et al.*(1993) detected Primiphos methyl in human diets in Lazio region of Italy. Yoshida *et al.*(1992) observed that the concentration of pesticide residue was greater in the fruit stalk, near the epidermis of fruits and in leafy vegetables. In Japan, Dieldrin and Heptachlor epoxide were detected in cucumbers and pumpkins, although their use is banned since 1972.

Joia *et al.*(1998) reported the possibility of pesticide residues in cauliflower grown in Punjab. The review suggests persistence of pesticide residues in soil and their uptake by crops for decades. In Singapore Goh *et al.*(1990) observed acute poisoning with Methamidophos (2.4 to 31.7 ppm) and Profenofos (1.1 to 5.4 ppm) residues in imported green leafy vegetable *Brassica alboglabra*. Nutley and Cocker(1993)

suggested that analysis of urinary Dialkylphosphate metabolites is useful for monitoring occupational exposure to organophosphorus pesticides and for detecting low levels of exposure which was not possible from cholinesterase inhibition method.

Kaur (1996) reported that Methyl parathion binds maximally to pepsin and casein, whereas, Monocrotophos binds maximally to lipase and bovine serum albumin (BSA). Bakshi (1996) reported that the binding of Malathion and Phenthoate to biomolecules/enzymes increased with increasing concentration of pesticide (50 to 450 μg a.i.). From the review it appears that more studies on the binding of organophosphorus pesticides to biomolecules and effect on their properties are needed. There is a need to devise simple inexpensive methods for extraction, detection and estimation of pesticide residues from foods/ tissues.

2.2 Toxicity of Organophosphorus Pesticides

The severity of toxicity of a pesticide depends on the factors like dose, the route of administration, the type of toxicity of the pesticide and its metabolites; extent of its accumulation and persistence in the body. Major routes of uptake of the pesticides are through skin, mucosa of respiratory and digestive tracts (Anon., 1990). The fat soluble pesticides are more efficiently absorbed through skin. For organophosphorus group of pesticides, the mechanism of acute toxicity to mammals has been well characterized as they inhibit cholinesterase(s) and affect the nervous system. The International Agency for Research on Cancer (IARC) concluded that there is "limited evidence" of carcinogenicity for humans

exposed to pesticides and this was mainly restricted to soft-tissue sarcomas. Contact dermatitis, allergic sensitization and photoallergic reactions have been observed in pesticide workers after exposure to Hexachlorobenzene, Benomyl and Zineb (Anon., 1990). Richards *et al.*(1996) reported jaundice in farmers due to dermal contact absorption, after dip treatment of sheep with organophosphorus pesticides. Bancroft (1993) implicated orgnophosphorus pesticides in the sexual problems of men working at farms in England. Sullivan (1993) also reported that pesticides affect reproduction and cause malformation in babies. A severity dependent inhibition of cholinesterase in RBC and plasma was observed with acute poisoning, especially during the initial stages (Bobba *et al.*, 1996). The neurotoxic syndrome was caused by Parathion, in upto 75% cases. Less than 10% of values of pseudocholinesterases indicate development of neurotoxic syndrome (Leon *et al.*, 1996). Neuro-psychiatric syndrome is caused by chronic exposure to organophosphorus pesticides (Ahmed and Davies, 1997). Kaur (1996) reported that Monocrotophos and Phorate produce extensive microsomal degranulation of rat liver cells under *in vitro* conditions and indicate carcinogenic potential. Randhawa (1991) reported that Methyl parathion and Ethion suppressed the secondary male characters with varying degree of atrophy of testes of pullets. Inhibition of spermatogenesis and degenerative cytoplasmic and nuclear changes in interstitial cells and maturing gametes was also observed. Verma and Ahuja (1998) observed similar results with Methyl parathion in male Wistar rats. Bertram (1991) reported that the epithelium of

gastro-intestinal tract is the first layer of host cells coming in contact with the ingested compounds (Fig.3). These cells respond earlier than other organs of the body by undergoing biochemical changes, including involvement of Cyt.P-450 pathways. Marver (1969) observed that drugs like phenobarbital and allyl isopropyl acetamide induce hepatic Cyt.P-450 but not Cyt.b5, due to induction of δ-aminolevulinic acid synthetase to provide increased amount of heme which in turn represses the drug mediated induction. Information about reproductive toxicity of currently used pesticides is not available in open published literature and this should be made available (Sullivan, 1993).

2.3 Microsomal Binding and Detoxification of Xenobiotics

The rat liver microsomes comprise of (Amar-Costesec *et al.*,1969) about 20% protein, 50% cholesterol and more than 50% of the RNA of liver homogenate (Fig.2). Compared to nuclear-, large granules- and supernatant fractions of the liver homogenate, the microsomal preparation contained 70-75% of the total microsomal material as judged from the activities of microsomal marker enzymes viz. glucose-6-phosphatase, esterase, nucleoside-diphosphatase and aminopyrine demethylase. Other enzymes viz. NADPH-cytochrome-C reductase were not exclusively of microsomal origin (Fig.2).

From Absorption Difference Spectra studies, Hodgson and Kulkarni (1974) studied the type of interactions between microsomes and xenobiotics (Table 1).

Remmer *et al.*(1969) studied the effect of various substrates on the Absolute Spectra of Cyt.P-450 of rat hepatic

microsomes. The Absorption Difference Spectrum showed two different effects (Fig.4). Type I compounds e.g. Hexobarbital decrease the intensity of Absolute Absorption Maxima at 420 nm and also shift it to 415 nm. Type II compounds e.g. Aniline shift the 420 nm Absolute Absorption Maxima to 426 nm. The hydroxylated aniline causes Type II spectral change. It agrees with both criteria, the magnitude of the spectral change and the content of Cyt.P-450. However, on comparing the rate of hydroxylation with Cyt.P-450 content, lack of correlation between these two criteria was indicated when hexobarbital or other Type I compounds were examined. When the rate of hydroxylation was compared with the magnitude of spectral change observed by Absorption Difference Spectroscopy, after the addition of hexobarbital, a good correlation was obtained, suggesting that some other factors were involved.

Sato *et al.*(1969) studied the Absolute Absorption Spectra of Cyt.P-450 (free from Cyt.b_5) of liver microsomes (Fig.4) from rabbits treated with Phenobarbital (80 mg kg^{-1} b.w. for 3 days, to induce the microsomal enzyme biosynthesis). Compared to oxidized Cyt.P-450, the Soret peak of those reduced with dithionite is broader, less intense and is at shorter wavelength of 412 nm instead of 416 nm. The deoxyhemoglobin shows Soret peak at 430 nm. The intensity ratio of the Soret peak to that of visible peak is considerably higher in deoxyhemoglobin than in reduced Cyt.P-450. In the visible region, one peak at 555 nm was present both in Cyt.P-450 and

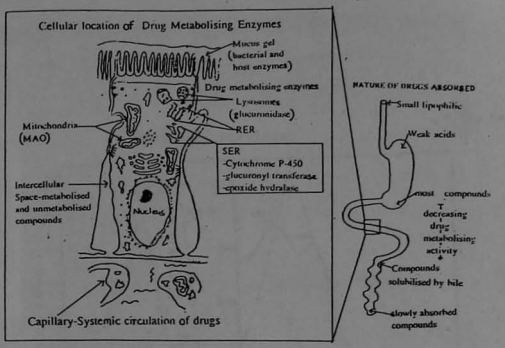


Fig. 3 Different sites and enzymes for biotransformation of compounds. (Modified from Bertram, 1991)

Table 1. Classification of Absorption Difference Spectra reported by Hodgson and Kulkarni (1974) and of present studies

Type	Peak(s) nm	Trough(s) nm	Site of interaction
Type I	385 (375-400)	420 (425)	Lipophillic site of microsomes away from heme iron of Cyt.P-450
Type II	430 (425)	390-410 (375-400)	Organic nitrogenous compounds bound to heme iron
Modified Type II or Reverse Type I	415-420 (425)	390 (375-400)	Nucleophillic oxygen to iron atom of heme; or displacement of an endogenous Type I substrate

Figures in the parentheses indicate modified absorption maxima which were adopted during the present studies.

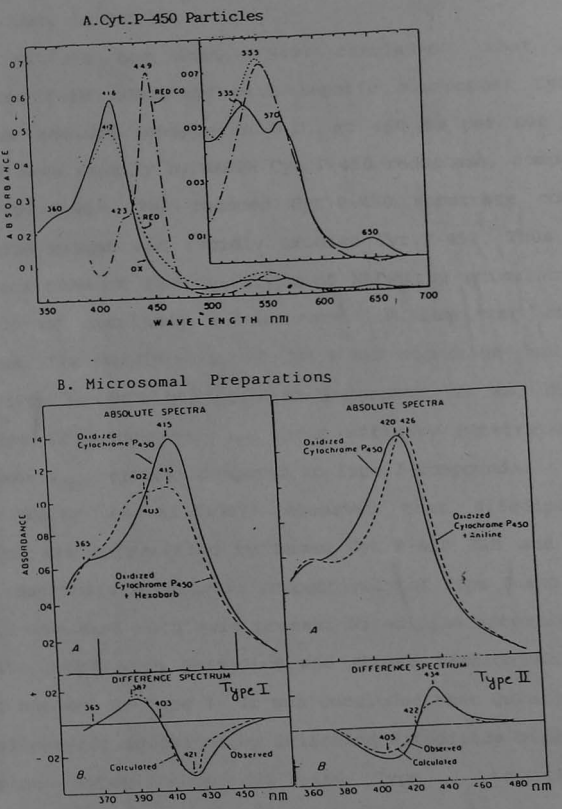


Fig.4. A: Absolute Absorption Spectra of Oxidised Cyt. P-450 Particles (———); reduced particles (– – – –); and reduced particles with CO (—·—·) (Adapted from Sato et al., 1969)

B : Absolute Absorption Spectra and Differences Spectra of hepatic microsomal suspensions respectively after addition of 10^{-4}M hexobarbital (Type I) and aniline (Type II) (Adapted from Remmer et al., 19969).

deoxyhemoglobin. It was concluded that reduction of Cyt.P-450 changes the structures around the heme group. The Soret peak of Cyt.P-450 coupled with CO was at 449 nm and the visible region peak was only at 555 nm.

Gillete and Gram (1969) concluded that Type I substrates form complexes with hepatic microsomal Cyt.P-450 which are reduced (change in O.D. at 450 nm per sec per mg protein) more readily by NADPH Cyt.P-450 reductase, compared to native Cyt.P-450. The reduced Cyt.P-450 substrate complexes react with oxygen and rapidly oxidise Cyt.P-450. Thus Type I substrates enhance the oxidation of NADPH by stimulating the reduction of complexes - the rate limiting step of these reactions. The deceleration of Cyt.P-450 reduction during Type II reaction is more difficult to interpret. It was concluded that Type II substances are poor affinity substrates (i.e. have lower V_{max} values) compared to Type I compounds.

Koley *et al.*(1997) observed that Nifedipine and Quinidine are metabolized by human Cyt.P-450 3AH and further showed that their binding is respectively of Type I and Type II to Cyt.P-450. When both were present Nifedipine interacted with Cyt.P-450 bound with Quinidine and showed binding changes of Type II but not to Type I. It was concluded that Quinidine acts as an allosteric inhibitor by switching Nifedipine binding from Nifedipine metabolising Cyt.P-450 Type I to the non-metabolizing Cyt.P-450 Type II.

Avocado (room ripened cv.Hass mesocarp tissue) microsomes show typical Type I Absorption Difference Spectra with Prosulfuron (Moreland *et al.*, 1996) and azoles (antifungal

agents) show Type II binding with *Candida albicans* microsomes. The target enzyme is Sterol 14α-demethylase (Venkateswarlu *et al.*, 1996a). Kahn *et al.* (1996) observed that microsomal Cyt. P-450 from etiolated seedlings of sorghum show Type I binding with Obtusifolial. The presence of Obtusifolial demethylase was shown in microsomes. Venkateswarlu *et al.* (1996b) reported that decreased Itraconazole-resistance of *Candida krusei* was not due to Type II binding to Cyt.P-450 Sterol 14α-demethylase but was due to decreased intracellular content i.e. decreased uptake of the drug.

Mehmood *et al.*(1995) reported that human Cyt.P-450 3AH, expressed in *Saccharomyces cerevisiae*, microsomes absorbed at 448 nm with herbicide Chlortoluron. The Cyt.P-450 3AH showed Type I binding spectrum and N-demethylation of the compound. Louw *et al.*(1997) administered (orally/i.p.) an analogue of Phenyl aziridine precursor 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethyl ammonium chloride (compound A) and elicited Type II Absorption Difference Spectrum but inhibited the Type I deoxycorticosterone induced Absorption Difference Spectrum of sheep adrenal Cyt.P-450 C11. Sheep and rat plasma attenuated this effect because compound A was stabilised by sex hormone binding globulin and corticosteroid binding globulin in plasma. Thus plasma proteins in *in vivo* may stabilize reactive compounds and keep them biologically active. Carelli *et al.* (1992) observed Type II binding of tetraconazole to Cyt.P-450 from fungus *Ustilago maydis*, indicating coordination between triazole N-4 and heme iron.

Mendis *et al.*(1992) reported that dithionite reduced

minus ferricyanide oxidised Absorption Difference Spectra of 600x g and 12000x g subcellular pellet fraction of adult male *Acanthocheilonema viteae* showed α-absorption maxima due to Cyt. C555, Cyt.b 562 and Cyt.aa3 (600-605 nm). The gamma Soret maxima of both fractions was at 427 nm, with a shoulder at 432-434 nm. Succinate reduced minus ferricyanide oxidised spectra of the 12000x g pellet mixed-sex adults of *A. viteae*, respectively showed λ max at 555 nm and 562 nm, 600 nm and 630 nm suggesting reduction of Cyt.C 555, Cyt.b562, Cyt.aa3 (600 nm) and an unidentified species at 630 nm. Antimycin A induced disappearance of 555 nm, 600 nm, 630 nm absorption maxima corresponding to Cyt.C 555, aa3 and an unidentified species. The 562 nm peak prevailed. These changes indicated functional involvement of Cyt.a, Cyt.b and Cyt.c.

Gaun *et al.*(1992) reported that fungitoxic sterol 14α-methylation inhibitors (DMIs) Imazalil, Itraconazole, Ketoconazole, Penconazole and Propiconazole interact with oxidised Cyt.P-450 isoenzymes, resulting in Type II binding Absorption Difference Spectra. Large N-1 substituents like Itraconazole and Ketoconazole did not show correlation between their displacement from Cyt.P-450 and fungal toxicity, whereas Imazalil, Penconazole and Propiconazole showed such relationship. Kapteyn *et al.*(1992) showed that the microsomal Cyt.P-450 isozymes of *Ustilago zeae* interact with sterol DMIs. The azole fungicides Prochloraz, Etaconazole, Imazalil, Triadimefon differ in toxicity to *U. zeae*. All these compounds induced Type II binding difference spectra at 10^9 to 10^8 M concentration. The IC_{50} (i.e. half saturation) of Type II do

not correlate with the fungicidal activities of azoles. Binding of CO to ferrous iron of heme of Cyt.P-450 was slightly inhibited by the DMIs tested but inhibition of CO binding did not correlate with fungitoxicity of DMIs. It was suggested that such studies are not useful for evaluating selective toxicity of DMI to intact sporidia of *U. zeae*.

Jeffery (1991) reported that Cyt.P-450 are a family of isoenzymes involved in oxidation and reduction of lipid soluble compounds. Cyt.P-450 content was highest in liver followed by that in kidney, lungs, gut – and nasal epithelial cells. Isoenzymes in hepatic endoplasmic reticulum (ER) showed little substrate specificity in oxidation or reduction reactions. More than 20 isoenzymes were identified. Cyt.P-450 dependent oxidation decreased lipid-water partition, decreased lipophilic storage and increased hydrophilic fraction in body water, leading to increased urinary excretion. Oxidative attack occurs at bonds involving N, S and C. The N-hydroxylation and aromatic oxidation (epoxide formation) were frequently associated with bioactivation to toxic intermediates. Detoxification and excretion were dependent on epoxide hydration or GSH conjugation.

Kyle and Farber (1991) reported that the Cyt.P-450 linked monooxygenase system is a heterogenous collection of enzymes and is responsible for biotransformation of foreign compounds. Cyt.P-450 refers to a series of related hemo-proteins, containing iron protoporphyrin as prosthetic group. Various forms of Cyt.P-450 have different apoproteins and show different specificities for their substrates. In association

with Cyt.P-450 the monooxygenase activity also requires NADPH dependent Cyt.P-450 reductase and Cyt.b5. The role of Cyt.b5 has not been established in Cyt.P-450 linked oxidations. The ER of hepatocytes contain highest content of Cyt.P-450. Ten distinct isozymes of Cyt.P-450 were identified in rat liver. Information about the role of Cyt.P-450 in farm animals has not been investigated to that extent.

Popp and Cattley (1991) observed that the exposure of liver to xenobiotics may result in non-toxic hepatic responses so as to adjust to the presence of xenobiotics e.g. Phenobarbital, produced 2-5 fold increase in smooth ER or Cyt.P-450 monooxygenase activity, leading to centrilobular hypertrophy. Part of this increase in SER may be related to impaired membrane catabolism. Such changes were reversed after withdrawl of xenobiotics. Herbicides may increase hepatic peroxisomes and associated enzymes upto 15 folds. In rodents such compounds produce centrilobular hypertrophy with granular cytoplasm. The SER inducers and peroxisome proliferators produced hepatic hyperplasia and hypertrophy. Such normal response was not associated with necrosis as shown by microscopy or serum enzyme alterations. A persistent increase in the liver weight, caused by prolonged treatment with chemicals that cause hyperplasia, may be associated with subsequent hepatocellular carcinogenicity.

Verma and Ahuja (1998) observed that 1/16 LD_{50}, 1/12 LD_{50} and 1/8 LD_{50} oral doses of Methyl parathion for 7 weeks increased the weight of brain, spleen, kidneys, testes and liver of rats which may be indicative of induction of enzymes

and carcinogenic tendencies of Methyl parathion.

Omura and Kuriyama (1969) reported that half life of rat hepatic microsomal proteins and phospholipids is 80h and 30-40h, respectively. There is rapid turnover of ER with constant enzyme profiles which indicate regulated synthesis and degradation processes. Drugs distort this regulation, leading to marked proliferation and altered composition of SER, especially NADPH Cyt.C reductase and Cyt.P-450 which are involved in drug metabolism. Phospholipid biosynthesis precedes those of enzymes/proteins; increase in reductase is earlier and higher than that of Cyt.P-450 mg^{-1} microsomal protein after exposure to Phenobarbital. There is increase both in weight of liver and amount of microsomes g^{-1} liver. Within 8d after last exposure to xenobiotics, the reductase activity returns to almost normal.

Bresnick and Madix (1969) reported that administration of 3-Methylcholanthrene elevates the aggregate RNA polymerase activity of liver. Nebert and Gelboin (1969) concluded that Phenobarbital, 3-Methylcholanthrene, Benz[a]anthracene, 7,12 Dimethylbenz[a]anthracene and Benz[a]pyrene induce microsomal hydroxylase involving Cyt.P-450 or its isoform. Aryl hydroxylase induction by 3-Methylcholanthrene is highest in small intestine followed by that in liver, kidney and lung. Pretreatment with Phenobarbital increased the microsomal protein biosynthesis.

Werck-Reichhart (1995) reported that Cyt.P-450 form a large family of heme-thiolate proteins catalysing Type I binding and metabolism of many xenobiotics. Cyt.P-450 catalysed

oxygenations results in detoxification and plays a role in resistance and selectivity to chemicals. Resistance to such chemicals may be correlated with modifications in the activity or expression of Cyt.P-450 oxygenases. Cyt.P-450 are highly inducible by chemicals. The specific tight binding or mechanism based inhibition is possible due to their unique catalytic properties. Scott and Roberts (1996) reported that Cyt.P-450 mediated desulfuration (activation) or dearylation (detoxification) in rat hepatic microsomes, which have greater capacity to detoxify and lower capacity to activate Chlorpyrifos, compared to Parathion. Numerous biochemical factors contribute to overall toxicity of Phosphorothionates. The *in vitro* efficiencies of hepatic microsomal desulfuration and dearylation of Chlorpyrifos and Parathion corresponds to acute toxicity.

Very little is known about the capacity of testes to activate and detoxify xenobiotics (Creasy and Foster, 1991). As their Cyt.P-450 and monooxygenase are important for the testicular steroidogenesis, so same enzymes may be involved in the metabolism of xenobiotics for toxicity/excretion. Leydig cells are the better sites of steroidogenesis than the Sertoli cells. The former lie outside the blood-tubule barrier. Both Leydig cells and Sertoli cells can be most susceptible to xenobiotics.

Moutou *et al.*(1998) studied the effects of 4-Quinolones, Oxolinic acid and Flumequine on hepatic microsomal cytochrome Cyt.P-450 monooxygenases in rainbow trout following oral antibiotic administration for 10 days. The

results of O-dealkylation, isoform-selective inhibition and immunoblotting showed that the effects of both Oxolinic acid and Flumequine were related to Cyt.P-450 1A subfamily. Cyt. P-450 binding spectra and *in vitro* experiments showed that both antibiotics are weak inhibitors (dose-independent) of Cyt.P-450 activity. Flumequine exhibited slightly higher binding affinity and inhibitory activity.

Johnston *et al.* (1994) studied the toxico kinetics interactions between the ergosterol-biosynthesis-inhibiting fungicides Prochloraz, Propionconazole and Penconazole and the organophosphorus insecticides Dimethoate, Chlorpyrifos, Diazuron and Malathion in a hybrid red-legged partridge (*Alectoris*). The tendency towards increased inhibition of serum BuChE activity by each of the organophosphorus pesticides in Prochloraz-treated birds was attributed to its active oxon form as a consequence of induction of microsomal monooxygenase by Prochloraz.

Padmanaban (1994) reported 27 families in the Cyt.P-450 gene superfamilies and subfamilies and reviewed the transcriptional regulation of the CYP2B1/B2 subfamily in rat liver by Phenobarbitone. Cyt.P-450 levels were higher in Chloroquine resistant compared to Chloroquine-sensitive strains of malarial parasites. Pretreatment of the parasite in culture with Phenobarbitone afforded partial protection to chloroquine toxicity, by induction of Cyt.P-450 which may sequester the drug or detoxify it. Nelson *et al.*(1993) characterized 12 putative pseudogenes out of 221 Cyt.P-450 genes. These genes have been described in 31 eukaryotes (including 11 mammalian

and 3 plant species) and 11 prokaryotes. Dubois *et al.*(1997) reported that DNA-adduct formation with genotoxic compounds was not same in the cultured hepatic cells of rodents, birds and human, possibly due to metabolic differences between the species. Each type of cultured cell, preferentially expressed different members of Cyt.P-450 families. In rat and human cells, Lindane strongly induced mRNA for Cyt.P-450, CYP2B and CYP3A, whereas Pentachlorophenol treatment induced those of Cyt.P-450, CYP1A, CYP2B and CYP3A. Dogra *et al.*(1998) reported that members of CYP1 Cyt.P-450 gene family are induced by polycyclic aromatic hydrocarbons. Cyt.P-450, CYP4 genes are activated by peroxisomal proliferators. The induction of Cyt.P-450 CYP2 gene family by Phenobarbital and that of CYP3 gene family by Dexamethasone appeared to involve the glucocorticoid receptor.

Cohen *et al.*(1994) investigated the sequence of a Cyt.P-450 encoding gene, CYP6A1 in an insecticide-resistant strain (Rutgers) and in susceptible strains (aabys and Sbo) of *Musca domestica*. The amino acid sequence of CYP6A1 is 98 per cent identical between Rutgers and susceptible strains. Differences in amino acid sequence occur in regions that do not participate in the active site.

2.4 Effects of Organophosphorus Pesticides on Carboxylesterases

Rat liver microsomes have five forms of carboxyl-esterases designated as A, B, C, S and egasyn (Yan *et al.*, 1995). Esterase-A and esterase-B respectively, hydrolyse the organophosphorus pesticides and are inhibited by pesticides (Aldridge, 1953) so their relative activities are of interest

to determine the pattern of selective toxicity.

Carboxylesterases A,B, C have mol.wt. of 57-59 kDa. Esterase-S, lacking C-terminal consensus sequence (HXE) was presumed to be secreted and had mol.wt. of 67-71 KDa. Deglycosylation of microsomes converted the 67-71 kDa protein to 57-59 kDa one. Liver and not the extrahepatic tissues are the source of serum carboxylesterase. Serum carboxylesterases are important in lipid metabolism and detoxification of organophosphorus pesticides. Secretion of hydrolase S and the modulation of its expression by xenobiotics may have physiological and toxicological significance (Yan *et al.*, 1995). Machin *et al.*(1975, 1978) reported that Coumaphos, Chlorpyrifos, Parathion and Diazinon are less toxic to mammals than to birds, because the former have higher activity of esterase-A in their blood plasma. Out of the 14 species of birds, the esterase-A activities in plasma were low, for Pyrimethyloxon and Paraoxon (Brealey *et al.*, 1980). The activities in mammals were 13 times higher than those in birds, so these pesticides will be less toxic to mammals. Compared to Paraoxon, Diazinon is hydrolysed more rapidly by esterase-A. Therefore, levels of esterase-A in plasma can help in predicting phylogenetic differences in selective toxicity of certain organophosphorus pesticides. Structure of pesticides influences its detoxification e.g. Carboxylesterase-A degrades Malathion (Sun *et al.*, 1992) but not many other organophosphorus pesticides.

Smyth *et al.*(1996) classified carboxylesterases by inhibition due to Paraoxon and Triphenyl phosphate. Thus

Malathion carboxylesterase in the Malathion-resistant high line might be structurally different from those in Malathion-susceptible, intermediate and low lines. Gopalan *et al.*(1997) reported that the elevated carboxylesterase had a greater affinity for alpha-naphthyl propionate compared to the acetate or butyrate esters.

Kimura *et al.*(1991) reported that the 200 kDa DCPA [chlorthal-dimethyl] hydrolysing carboxylesterase is made up of homo-oligomeric 6 kDa subunits as determined by gel filtration and SDS-PAGE, respectively. By using isoelectric focussing, Sakata and Miyata (1994) identified several carboxylesterases in plant hopper (*L. striatella*) resistant to Malathion. These esterases had polymorphic characteristics and their molecular weights ranged between 66 to 70 kDa, due to variation in glycosylation. The pI values of these esterases ranged from 5.3 to 4.7. Newcomb *et al.*(1997) reported that a single amino acid substitution of neutral to acidic one (Gly 137 → Asp) at the active site of enzyme converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance in blowfly (*Lucila cuprina*). Sun and Chen (1993) suggested that the carboxylesterase serves both as a catalytic protein for the hydrolysis of some insecticides (e.g. Malathion and trans-Permethrin) and a binding protein for the oxons of the several organophosphorus compounds, some Carbamates and Pyrethroids. It was proposed that gene encoding the enzymes was expressed to a higher extent in resistant compared to that in susceptible plant hoppers. Thompson *et al.*(1991) observed increase in the activity of avian serum esterases following exposure to low

dose of Demeton-S-methyl and with higher doses inhibition was observed. Brondeau *et al.*(1991) compared the activities of Malathion carboxylesterase in serum with those of glucose-6-phosphatase as indicator of damage to ER; and of serum glutamate dehydrogenase and sorbitol dehydrogenase as indicator of hepatic cytolysis. Chambers and Chambers (1990) studied the time course inhibition of acetylcholinesterase and aliesterases following exposures of rats to Parathion and Paraoxon. In all groups, the brain acetylcholinesterase was partially inhibited before the hepatic carboxylesterases. The plasma carboxylesterases were maximally inhibited within 15 min after administration of organophosphorus insecticide

Awal *et al*(1994) studied the effects of long-term exposure to Anthio on serum esterase in male calves. The serum cholinesterase and carboxylesterase were inhibited in the groups getting 6 mg per kg b.w.(10-28%) and 12 mg per kg b.w. (10-33%).

Raina *et al.*(1990) studied the influence of repeated oral administration of Dichlorvos on circulating esterases in buffalo calves. Maximum inhibition of plasma cholinesterase (87-94%) and serum carboxyl esterase (51-67%) was observed on 28th day. An increase in total plasma protein was also observed. Ehrich *et al.*(1993) reported that the activities of brain-and blood cholinesterase and carboxylesterase were more sensitive to inhibition by Diisopropyl phosphorofluoridate than the hepatic enzymes. Awal (1992) studied the toxicity of single topical application of Dichlorvos in male calves. The extent of inactivation of blood esterases did not correlate with the

severity of toxicity. Up to 6% inhibition of blood cholinesterases was detected on 21 days after the administration of Dichlorvos. Fossi *et al.*(1994) suggested that the estimation of inhibition of blood esterase in birds serves as an index of organophosphorus contamination of the fields frequented by the birds.

Huang and Huang (1989) reported that Malathion-carboxylesterase can hydrolyse Malathion and other compounds involving carboxylic bond, but not the phosphate ester bond and contributes to resistance in *Culex pipiens* towards the insecticides, by its absorptive/sequestering effect. Yang *et al.*(1988) suggested that the increased carboxylesterase activity may be one of the mechanisms causing high resistance of *Culex tritaeniorhynchus* to organophosphorus insecticides. Ketterman *et al.*(1992) purified and characterized carboxylesterase, involved in insecticide resistance, from mosquito *C. quinaquefasciatus*. The kinetic constants for Temephos, Chlorpyrifos, Fenitrothion and Propoxur, indicated that the rates of acylation and the affinities of binding of the insecticides to this carboxylesterase are important. This suggests that carboxylesterase sequesters the insecticides. Suzuki *et al.*(1993) also reported that carboxylesterase acts as a sequestering protein during Fenitrothion resistance.

Whyard *et al.*(1994) purified an esterase, conferring insecticide resistance from the mosquito *Culex tarsalis*. The Malathion-carboxylesterase is expressed throughout the development and is most concentrated in the gut tissue of larvae. Malathion carboxylesterase was localized in the

mitochondria and cytoplasm of resistant and susceptible insects, respectively. Chromatofocussing indicated that resistant insects have two Malathion carboxylesterases with pI of 6.8 and 6.2, while susceptible insects possessed the one with a pI of 6.8. Thus the resistance in *C. tarsalis* is due to the presence of a qualitatively different esterase in the resistant strain.

Small *et al.*(1998) reported that amplification of esterases was the major mechanism of inducing resistance in *Culex* mosquitoes. The selective advantage of the Est alpha 21/ Est.beta21 Amplicon was not due to its greater efficiency for binding to insecticide as organophosphorus insecticides were significantly better inhibitors of all the amplified esterases compared to their non-amplified counterparts and are, therefore, equally effective in conferring resistance.

From the correlation between Diazinon resistance and Aldrin epoxidation Kotze and Sales (1995) suggested that both the carboxylesterase and monooxygenase systems may contribute to Diazinon resistance. The latter is the basis of organophosphorus pesticide resistance in larvae of blowfly affecting Australian sheep. Boone and Chambers (1996) reported that in mosquito fish (*Gambusia affinis*) non-protein sulfhydryl group concentration was significantly decreased after 24h by all compounds, particularly Parathion-methyl. The protection of carboxylesterase from the effects of these insecticides may be provided by hepatic aliesterases. For Parathion-methyl this protection is much less.

From the review of literature it appears that there is a need to develop quick and cheap methods for extraction/estimation of pesticide residues as well as for understanding the molecular basis of variation in toxicity of organophosphorus pesticides in different tissues of farm animals.

CHAPTER III

MATERIAL AND METHODS

Simple, inexpensive methods for extraction, detection and estimation of pesticide residues from food/tissues are not available. Thin layer chromatography for fractionation of pesticides, the detection of the components of organophosphorus pesticide with Lowry reagents was carried out to determine the lower limits of their detection and estimation. To explain the mode of action of these pesticides, their binding to enzymes and microsomes as well as inhibition of digestive and microsomal esterases of tissues of goat were investigated. The details of the methodology used is given below:

3.1 Sources and Characteristics of Pesticides

The pesticides (Fig.5, Table 3), Ethion (EC 50%), Phorate (10% w/w), Phenthoate (EC 50%), Malathion (EC 50%), Methyl-parathion (EC 50%), Quinalphos (EC 25%), Dimethoate (EC 30%) and Monocrotophos (36% w/w) a gift from Northern Minerals Ltd., Gurgaon, India, were used during the present studies. The 50 μg to 250 μg concentrations of each pesticide used and μg to μM relationship of each pesticide are also given in Table 2.

Table 2. Purity, multiplication factors (for estimation of pesticide from phosphorus content) and relationship between μg and μM of different pesticides

	Ethion	Phorate	Phenthoate	Malathion	Methyl parathion	Quinalphos	Dimethoate	Monocrotoph
Mol.wt.	384	260	320	330	263	298	229	223
Multiplication factor	6.19	8.38	10.32	10.65	8.48	9.61	7.38	7.19
Purity(% a.i.) Calculated	50	10	50	50	50	25	30	36
*Estimated	49.1	9.0	42.5	53.5	56.5	34.4	42.7	44.0
a.i.(μg)				**a.i.(μM)**				
50	.13	.14	.16	.15	.19	.17	.22	.22
100	.26	.28	.31	.30	.38	.34	.44	.45
150	.39	.42	.47	.45	.57	.50	.66	.67
200	.52	.56	.63	.61	.76	.67	.87	.90
250	.65	.69	.78	.78	.95	.84	1.09	1.12

*Estimated gravimetrically after acetone extraction of the commercial pesticide

Table 3. Comparative characteristics of pesticides used (Martin, 1968)

Pesticide	Oral LD_{50} (mg a.i. kg^{-1} b.w.)	Properties
Ethion (EC 50%) (OO,O'O'-tetraethyl SS'-methylene diphosphorodithioate)	208	Sparingly soluble in water, soluble in most organic solvents, slowly oxidised and hydrolysed by acids and alkalies
Phorate (10% w/w) (OO-diethyl S-ethyl-thiomethyl phosphoro-dithioate) granules	1.6-3.7	Miscible with carbon tetra-chloride, dioxon, vegetable oils, susceptible to alkali
Phenthoate (EC 50%) (S-α-ethoxycarbonyl benzyl O,O-dimethyl phosphorodithioate)	300-400	Miscible with organic solvents, stable in acidic, neutral and alkaline media
Malathion (EC 50%) (S-1, 2-di(ethoxy-carbonyl) ethyl OO-dimethyl phosphoro-dithioate)	2800	Miscible with organic solvents, stable in pH 5.0 to 7.0
Methylparathion (EC 50%) (OO-dimethyl O-4-nitrophenyl phosphorothioate)	14-24	Slightly soluble in mineral oils, soluble in most organic solvents, susceptible to alkali, a good methylating agent
Dimethoate (EC 30%) (OO-dimethyl-S-methyl carbamoyl methyl phosphorodithioate)	500-600	Soluble in organic solvents except saturated hydrocarbons susceptible to alkali
Quinalphos (EC 25%) (OO-diethyl-O-quinoxalin-2-yl-phosphorothioate)	62-137	Soluble in ethanol, methanol, ketones, aromatic hydro-carbons, susceptible to acid
Monocrotophos (EC 36%) (Dimethyl cis-1-methyl-2-methyl carbamoyl vinyl phosphate)	8-23	Miscible with water, soluble in acetone, ethanol, susceptible to alkali

Values in parentheses indicate active ingredient (a.i.) content

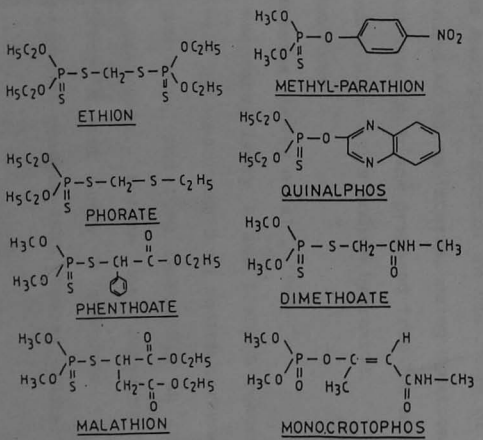


FIG. 5. STRUCTURES OF DIFFERENT ORGANOPHOSPHORUS PESTICIDES

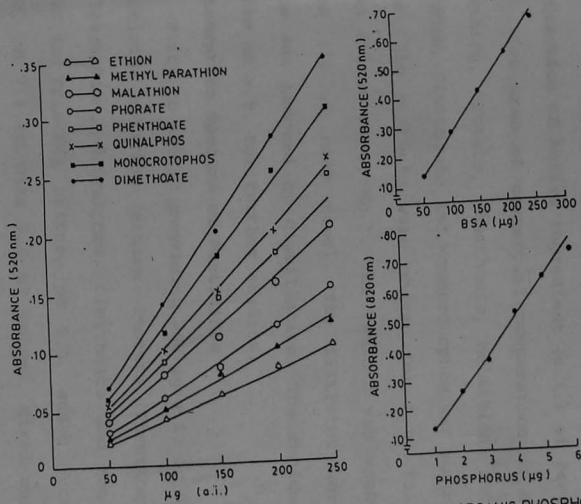


FIG. 6. STANDARD CURVES FOR ESTIMATION OF PROTEINS, IN ORGANIC PHOSPHORUS AND ORGANOPHOSPHORUS PESTICIDES.

3.2 Extraction of Active Ingredient (a.i.) of Pesticides

Ten gram of commercial preparation of each pesticide viz. Ethion, Phorate, Phenthoate, Malathion, Methylparathion, Quinalphos, Dimethoate and Monocrotophos was suspended in 30 ml acetone. The contents were shaken for 1 h, filtered through Whatman filter paper No.1. The residue was washed twice with acetone (15 ml each time). The filtrates were pooled and acetone was removed by distillation. The residue was weighed and used as a.i. for further studies.

3.3 Absorption Spectra of Pesticides

Fifty to 100 μl (providing 250 μg a.i. or 0.65μM-1.12 μM) of pesticide solution (Ethion, Phorate, Phenthoate, Malathion, Methylparathion, Quinalphos, Dimethoate and Monocrotophos) in 1% DOC in Tris-HCl buffer, pH 8.0, added Sucrose-Tris (ST) buffer, pH 7.4 to make 2.0 ml volume. Mixed and incubated at 26°C for 15 min, added 2.0 ml 0.05N sodium hydroxide to each tube. The Absorption Spectra in the range of 350 nm to 575 nm were recorded at 10 nm interval using Spectronic 20 (Bausch and Lomb, USA) against blank prepared from 2.0 ml ST buffer, pH 7.4 mixed with 2.0 ml 0.05N sodium hydroxide.

3.4 Estimations

3.4.1 Proteins (Lowry *et al.*, 1951)

3.4.1.1 Reagents: Lowry A and Lowry B reagents were prepared respectively by dissolving 2.0g sodium carbonate in 100 ml 0.1N sodium hydroxide, and 1.0g $CuSO_4.5H_2O$ in 100 ml 1.0% sodium citrate; immediately before use, reagent C was prepared by mixing Lowry A and Lowry B reagents (50:1, v/v). Folin-Ciocalteu phenol reagent (SRL, Bombay, India) was diluted (1:1,

v/v) with distilled water.

3.4.1.2 Method: To various concentrations (50 to 250 μg in 250 μl) of BSA, added 2.5 ml of reagent C, vortexed, kept at room temperature for 10 min, added 250 μl diluted Folin-Ciocalteu Phenol reagent to each tube and vortexed again. After 30 min at room temperature, read the absorbance at 520 nm by using Spectronic 20 (Bausch and Lomb, USA). A standard curve was prepared (Fig.6).

3.4.2 RNA (Munro and Fleck, 1966)

To 0.1 ml microsomal preparation, added 2.5 ml 0.22N cold perchloric acid, cooled in ice for 10 min, centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the residue was made moisture free by inversion of the tubes, for 5 min, over a filter paper. Added 3.2 ml 0.3N KOH, vortexed and incubated at 37°C for 60 min. Added 0.8 ml cold 2.2N perchloric acid to each tube, kept in ice for 5 min and centrifuged for 5 min at 3000 rpm (using Centrifuge Remi R8C, India). The supernatant was diluted (1:1 v/v) with distilled water and the absorbance was measured in quartz cuvettes, at 260 nm against a water blank in a UV/Vis Spectrophotometer (Model DU-7, Beckman, USA). RNA concentration was calculated from the following equation:

0.1 AU at 260 nm = 256μg RNA ml^{-1} in the original suspension

3.4.3 Estimation of Pesticides by Lowry Reagents for proteins

The pesticides and their components were estimated on the basis of their phosphorus content as determined by Ames (1966) method for inorganic phosphorus and on the basis of intensity of absorption after reaction with Lowry reagents (Bakshi, 1996) as used for estimation of proteins (Lowry et

al., 1951). This procedure was adopted and separate standard curves for Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos were prepared using 50 to 250 μg a.i. of each pesticide.

3.4.3.1 Method: Ten to 100 μl (providing 50 μg-250 μg a.i. or 0.13 μM - 1.12 μM) of a pesticide solution in 1% DOC in Tris-HCl buffer, pH 8.0 (Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos) were processed to develop colour and recorded the absorbance as described for proteins. As different pesticides showed variable absorbance so separate standard curves (Fig.6) for a.i. of each pesticide were prepared.

3.4.4 Phosphorus (Ames, 1966)

To the sample (a.i. of pesticides or their fractions) added 0.18 ml of 10% magnesium nitrate in 95% aqueous ethanol. Heated to dryness at 100°C and oxidised on direct flame or an electric heater, till samples turned white and ceased to release brown fumes. Cooled and added 1.8 ml 0.5N HCl to each tube. Heated for 15 min under condensation by using glass-ball condensers, in boiling water bath. Cooled and added 4.2 ml colour developing reagent (0.42% ammonium molybdate in 1N H_2SO_4 and 10% ascorbic acid - 6:1, v/v). Incubated in hot water bath at 45°C for 20 min. Recorded the absorbance at 820 nm against a blank prepared without the sample. A standard curve (Fig.6) was prepared by using KH_2PO_4 solution (stock solution 5 mg ml^{-1}) from which working solutions providing 1-6 μg phosphorus were prepared. From the phosphorus content, the content of each pesticide was calculated by using various factors (Table 2).

3.5 Detection of Organophosphorus Pesticides Fractionated by TLC (Tomizawa, 1976)

3.5.1 Preparation of TLC plates:

The glass plates (20 cm x 20 cm or 5 cm x 20 cm) were washed thoroughly, cleaned with acetone and oven dried. A slurry of self binding silica gel H (Acme's Laboratory Chemicals, Bombay, India) for TLC was prepared by mixing 7.0g gel with 21.0 ml distilled water, for 20 cm x 20 cm plates and 2.5g gel in 7.0 ml distilled water for 5 cm x 20 cm plates. The slurry was poured on to the centre of the plate, spread uniformly with a glass rod, gently tapped the plate and allowed the gel to dry at room temperature, and on a levelled surface. Before use, the dried gel coated plates were activated at 110°C for 45 min and cooled in a desiccator. The pesticides were fractionated, visualized and the Rf value of each component was recorded.

3.5.2 Fractionation of Pesticides:

Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos were fractionated by TLC on silica gel H coated plates by using the solvent system Benzene: Ethyl Methyl Ketone (5:3, v/v). The TLC chamber was lined with Whatman No.1 filter paper, covered and allowed to saturate with the solvent. The samples were applied with capillary tubing (above the meniscus of the solvent system) with intermittent drying to limit the diameter of sample spots. After ascending chromatography upto 16 cm from the point of application, marked the position of solvent front, removed and air dried the plates. The fractionated components were visualised with iodine vapour and marked to determine the

Rf values of each component. For preparative purposes, the bands were scrapped, eluted and the components were estimated to determine the proportion of each component.

3.5.3 Detection of Pesticide Components: Components of the eight pesticides fractionated by TLC were visualized with the following reagents:

3.5.3.1 Iodine vapour (Sims and Larose, 1962): The iodine crystals in glass crucible were warmed and kept in the chamber with the TLC plate. The components appeared yellow brown. These were marked, the Rf values were measured and for preparative purpose the visualised components were quantitatively scrapped (pooled with acetone washings of glass surface) and eluted with acetone. The acetone extract was filtered through G-3 sintered glass funnel and evaporated to remove acetone. The proportion of each component was measured gravimetrically and also by Ames (1966) method for phosphorus. The pesticide content was calculated by using the factors given in Table 2.

3.5.3.2 Lowry Reagents

Bakshi (1996) observed that different organophosphorus pesticides give colour of measurable absorbance at 520 nm with Lowry reagents, as used for estimation of proteins (Lowry *et al.*, 1951). Therefore, these reagents were used for detection and estimation of pesticides and of their fractionated components. Immediately after evaporation of solvent the developed plates were sprayed with Lowry reagent C (subhead 3.4.1) as described for estimation of proteins and left at room temperature for 15 min. Any coloured adduct formed was noted. The plates were then sprayed with Folin-Ciocalteu phenol (1:1, v/v) reagent. With

some exceptions all the fractionated components appeared blue. The intensity of blue colour was maximum after 1 h and was stable for atleast one week.

3.5.3.3 Cupric Acetate-Phosphoric Acid Reagent (Fewster *et al.*, 1969): The reagent was prepared by dissolving 3.0g cupric acetate in 8.0% aqueous phosphoric acid. The developed TLC plates were uniformly sprayed and heated at 150°C for 30 min. Some of the components of the eight pesticides gave different colours, representing formation of different adducts and others turned black on heating. At this stage, colour of all components were recorded again. The characteristics of distinct components (if any for a pesticide) fractionated by TLC and visualized by Lowry reagent or cupric acetate-phosphoric acid reagents were recorded. To determine the detection limits of the spray reagents different amounts (3.12, 6.25, 12.5, 25, 50 and 100 μg or ppm) of pesticides were fractionated and visualized, as described above.

3.5.4 Binding of Pesticides to Trypsin

3.5.4.1 Reagents: Prepared 10 mg ml^{-1} solution of commercial trypsin (2 units mg^{-1}, SRL, Bombay, India) in PBS, pH 7.4.

3.5.4.2 Method: To 50 μl to 100 μl (providing 250 μg a.i.) of each pesticide (Ethion, Phorate, Phenthoate, Malathion, Methyl-parathion, Quinalphos, Dimethoate and Monocrotophos) was taken and made upto 100 μl with 1% DOC in Tris-HCl buffer, pH 8.0. Mixed with 100 μl trypsin solution containing 1 mg trypsin and incubated at 37°C for 15 min. After incubation, added 2 ml (10 volumes) of chloroform, vortexed, allowed to separate into two clear layers, collected the lower chloroform layer containing

the pesticide residues not bound to trypsin. The chloroform free residue was weighed and fractionated by TLC to identify the a.i. of pesticide(s) which did not bind to trypsin. The fractionation was done in Benzene:Ethyl Methyl Ketone (5:3, v/v) solvent system. The same amount of a.i. extracted with acetone from commercial preparation was also run for comparison.

3.6 Absorption Spectra of Organophosphorus Pesticides Comlexed with Lowry Reagents

3.6.1 Method: Took 50 to 100 μl (providing 250 μg a.i. or 0.65 μM - 1.12 μM) of pesticide solution in 1% DOC in Tris-HCl buffer, pH 8.0. Developed the colour as described for proteins. Recorded the absorbance of colour complexes between 360 nm to 730 nm at 10 nm intervals using Spectronic 20 (Bausch and Lomb, USA). This was done to determine the λ max of colour complexes formed by each pesticide. The same was used to prepare different standard curves for estimation of pesticides or their components (Fig.6).

3.7 Partial Purification of Microsomes

3.7.1 Collection and Processing of Tissues

Liver, spleen, brain, kidneys, testes, abomasum and duodenum were collected from freshly slaughtered goats through a local meat shop. These tissues were freed from adventitious tissues and washed with cold sodium chloride (0.9%) solution.

3.7.1.1 Homogenisation of Tissues: The organs/tissues were swab dried on filter paper, weighed, finely chopped and homogenised (except abomasum and duodenum) with 0.225M ST-buffer, pH 7.4 (2.5ml buffer g^{-1} tissue) prepared by dissolving 19.56g sucrose and 0.756 g tris in 150 ml distilled water, adjusted to pH 7.4

with 1N HCl and made up the volume to 250 ml under cold conditions.

The abomasum and duodenum (60 cm length from abomasum) of the goat was dissected out, opened and washed with water to get rid of digesta and then cleaned with 0.9% sodium chloride. These tissues were swab dried on filter paper, weighed and the mucosa was scrapped with the help of clean, microscope glass slide, in ST buffer, pH 7.4. The scrapped mucosal tissue was weighed and used for preparation of calcium chloride precipitable proteins. The rumen was also obtained, cleaned but the papillae were very hard and could not be scrapped with the glass slide. So these were cut out with the help of a surgical blade, even then it was difficult to homogenize them. So rumen papillae were not processed further.

3.7.2 Partial Purification and Binding of Pesticides to Microsomes

The microsomes were prepared from liver, brain, spleen, kidneys and testes of goats. The extent and type of binding of Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos to microsomes of tissues of goats were studied. For abomasal and duodenal scrapping, the homogenization was omitted.

3.7.2.1 Preparation of Microsomes (Kamath and Narayan, 1972 as modified by Gupta and Dani, 1979): Goat liver, brain, spleen, kidneys and testes (50 g each) were suspended in 0.9% cold sodium chloride solution, swab dried on filter paper, weighed and finely chopped with sharp blade and suspended in 0.255M ST buffer, pH 7.4. These were homogenized at 4°C by keeping ice in

a beaker around the homogeniser tube in 2.5 volumes of ST buffer for each gram of tissue by using a motor driven Potter-Elvehjem homogeniser having teflon pestle (U.P. National Mfrs. Pvt. Ltd., Varanasi, India). After 10-12 passes the homogenate was centrifuged for 20 min at 9000 rpm in fixed angle rotor at 4°C in cold centrifuge (Model C24, Remi, India). The post mitochondrial supernatant was mixed with 0.8M calcium chloride ($CaCl_2.2H_2O$) to get 8 mM final concentration of calcium chloride. The tubes were kept in ice for 20 min, centrifuged again for 20 min at 10,000 rpm and at 4°C. The pelleted microsomes were suspended in 0.225M ST buffer, pH 7.4. The ratio of RNA to protein in microsomal preparation was determined. From the absorbance of RNA at 260 nm against a water blank in a UV/vis spectrophotometer (Model DU-7, Beckman, USA) and estimation of proteins by Lowry *et al.*(1951), the ratio of RNA:protein was calculated (Table 4).

Table 4. Composition of microsomal preparation from tissues of goats

Tissue	RNA (mg ml^{-1})	Protein (mg ml^{-1})	RNA:Protein
Liver	0.105	12.90	0.008
Kidney	0.180	10.16	0.011
Testes	0.690	7.95	0.086
Brain	0.480	4.02	0.119
Spleen	0.115	7.95	0.014
Abomasal mucosa*	0.510	5.63	0.091
Duodenal mucosa*	0.280	5.36	0.052

*Represent calcium chloride precipitable RNA-protein complexes from cells of these tissues

3.8 Binding of Pesticides to Biomolecules

3.8.1 Pepsin

3.8.1.1 Reagents: Prepared 10 mg ml^{-1} solution of commercial pepsin(3000 units mg^{-1}, extrapure, SRL, Bombay, India) in 0.01N HCl. The pesticides (Ethion, Phorate, Phenthoate, Malathion, Methyl-parathion, Quinalphos, Dimethoate and Monocrotophos), dissolved in 1% DOC in Tris-HCl buffer, pH 8.0 were added in varying volumes (10 to 100 μl) to get 50 to 250 μg a.i. or 0.13 μM - 1.12 μM concentration of the pesticide.

3.8.1.2 Method: Ten to 100 μl (providing 50 to 250 μg a.i. or 0.13 μM - 1.12 μM pesticide) solution of each pesticide was made upto 100 μl with 1% DOC in Tris-HCl buffer, pH 8.0. Mixed with 0.1 ml (1 mg) pepsin solution and incubated at 37°C for 15 min. Added $\geq$ 2 ml (10 volumes) of chloroform, vortexed, allowed to form 2 layers, collected the lower chloroform layer containing pesticide constituent(s) not bound to pepsin processed to estimate pesticide(as explained in subhead 3.4.3). The amount of pesticide bound to enzyme was calculated as follows and expressed as per cent pesticide(s) bound to pepsin:

$$\left[100 - \frac{\text{Pesticide}(\mu g \text{ a.i.}) \text{extracted}}{\text{Total pesticide}(\mu g \text{ a.i.}) \text{added}}\right] \text{ X } 100$$

3.8.2 Trypsin

3.8.2.1 Reagents: Prepared 10 mg ml^{-1} solution of commercial trypsin (2 units mg^{-1}, SRL, Bombay, India) in PBS, pH 7.4. PBS was prepared by dissolving 16.0g sodium chloride, 0.4g potassium chloride 0.4g KH_2PO_4, 0.4g sodium azide and 2.882g $Na_2HPO_4.2H_2O$ in distilled water and made up the final volume to

2.0 litres.

3.8.2.2 Method: Ten to 100 µl (50 to 250 µg or 0.13 µM - 1.12 µM a.i.pesticide) solution of each pesticide was made upto 100 µl with 1% DOC in Tris-HCl buffer, pH 8.0. Mixed and incubated with 0.1 ml (=1 mg) of trypsin solution and incubated at 37°C for 15 min. Added 2 ml (10 volumes) of chloroform, vortexed, allowed to form 2 layers, collected the lower chloroform layer containing the pesticide constituent(s) not bound to trypsin, estimated and calculated the pesticides as described for pepsin (subheads 3.4.3 and 3.8.1).

3.8.3 Lipase

3.8.3.1 Reagents: Dissolved 100 mg commercial lipase (>7300 Wilson units g^{-1}, Calbiochem, USA) B grade from Porcine pancreas in 2 ml Tris-HCl buffer, pH 8.5.

3.8.3.2 Method: Ten to 100 µl (providing 50 to 250 µg or 0.13 µM - 1.12 µM a.i.) solution of each pesticide was made upto 100 µl with 1% DOC in Tris-HCl buffer, pH 8.0. Mixed with 0.2 ml (=10 mg protein) lipase solution and incubated at 37°C for 15 min. Added 3 ml (10 volumes) of chloroform, vortexed, allowed to form 2 layers and collected the lower chloroform layer containing the pesticide constituent(s) not bound to lipase, estimated and calculated the pesticides as described for pepsin (subheads 3.4.3 and 3.8.1).

3.8.4 Bovine serum albumin (BSA)

3.8.4.1 Reagents: To 8 ml of 2.5% BSA in 0.3N HCl, added 1.5 ml 0.3N HCl. Warmed at 37°C for 5 min. Made up the volume to 10 ml with 0.3N HCl to get 20 mg BSA ml^{-1}.

3.8.4.2 Method: Ten to 100 µl (50 to 250 µg or 0.13 µM - 1.12

μM a.i.pesticide) solution of each pesticide was made upto 100 μl with 1% DOC in Tris-HCl buffer, pH 8.0. Incubated with 0.1 ml (=2 mg) BSA solution at 37°C for 15 min. Added 2 ml (10 volumes) of chloroform, mixed, separated into 2 layers, collected the lower chloroform layer containing the pesticide constituent(s) not bound to BSA, estimated and calculated the pesticides as described for pepsin (subheads 3.4.3 and 3.8.1).

3.8.5 Casein

3.8.5.1 Reagents: Added 50 mg casein (SRL, Bombay, India) to 1 ml Tris-HCl buffer, pH 9.2 and stirred on magnetic stirrer for 2h, centrifuged at 3000 rpm for 10 min and the protein content of the supernatant was determined to be 23 mg ml^{-1} indicating that 46 per cent of the casein was dissolved.

3.8.5.2 Method: Ten to 100 μl (50 to 250 μg or 0.13 μM - 1.12 μM a.i.pesticide) solution of each pesticide was made upto 100 μl with 1% DOC in Tris-HCl buffer pH 8.0. Mixed and incubated with 0.1 ml (=2.3 mg) casein solution at 37°C for 15 min. Added 2 ml (10 volumes) of chloroform mixed allowed to form 2 layers and collected and evaporated the lower chloroform layer containing the pesticide constituent(s) not bound to casein, estimated and calculated the pesticide as described for pepsin (subheads 3.4.3 and 3.8.1).

3.8.6 Sucrose

3.8.6.1 Reagents: Sucrose 34 mg (Himedia Laboratories, Bombay, India) was dissolved in 2 ml distilled water.

3.8.6.2 Method: Ten to 100 μl (providing 50 to 250 μg or 0.13 μM - 1.12 μM a.i.) solution of each pesticide was made upto 100 μl with 1% DOC in Tris-HCl buffer, pH 8.0. Mixed and incubated

with 0.2 ml (=0.34 mg) sucrose at 37°C for 15 min. Added 3 ml (10 volumes) of chloroform, mixed allowed to form 2 layers, collected and evaporated the lower chloroform layer containing the pesticide constituent(s), not bound to sucrose, estimated and calculated the pesticide as described for pepsin (subheads 3.4.3 and 3.8.1).

3.9 Binding of Pesticides to Microsomes

The microsomes prepared from liver, spleen, brain, kidneys and testes of goats were suspended in 50 ml ST buffer, pH 7.4 (1.0 g tissue ml^{-1}). To 1.8 ml of microsomal preparation added 50-100 μl (providing 250 μg a.i. or 0.65 μM - 1.12 μM a.i) of pesticide solutions in 1% DOC in Tris-HCl buffer, pH 8.0 and made up the final volume to 2 ml with ST buffer, pH 7.4. In the control tubes, added 0.2 ml of 0.225M ST buffer, pH 7.4 in place of pesticides. Incubated at 26°C for 15 min, added 5 ml chloroform to each tube, mixed, allowed to separate into two clear layers and collected the lower clear chloroform layer. Estimated the unbound pesticide as described for pepsin (subheads 3.4.3 and 3.8.1). The per cent pesticide bound to microsomal preparation was calculated:

$$\left[100 - \frac{\text{Pesticide } (\mu g \text{ a.i.}) \text{ extracted}}{\text{Pesticide } (\mu g \text{ a.i.}) \text{ added}}\right] \times 100$$

3.10 Absolute and Difference Absorption Spectra of Microsomes and Their Complexes with Pesticides

For Absolute Absorption Spectral studies, took 1.8 ml suspension of microsomal preparations of liver, spleen, brain, kidneys and testes, respectively representing the protein

content of 14.06, 7.03 7.81, 5.94 and 5.00 mg ml^{-1}. Added 50-100 μl (providing 250 μg or 0.65 μM - 1.12 μM a.i.) of pesticide (Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos) dissolved in 1% DOC in Tris-HCl buffer, pH 8.0. Made up the final volume to 2 ml with ST buffer, pH 7.4. Mixed and incubated at 26°C for 15 min, added 2.0 ml 0.05N sodium hydroxide to each tube. The control tubes did not contain pesticides but were prepared by mixing 1.8 ml microsomal preparations from liver, spleen, brain, kidneys and testes with 0.2 ml ST buffer, pH 7.4 and 2.0 ml 0.05N sodium hydroxide. The Absorption Spectra in the range of 350 nm to 575 nm were recorded at 25 nm interval using Spectronic 20 (Bausch and Lomb, USA) against blank prepared from 2.0 ml ST buffer, pH 7.4 and 2.0 ml 0.05N sodium hydroxide.

Absorption Difference Spectra of microsomes and their complexes with pesticides (modified from Remmer *et al.*, 1969) were plotted by calculating the change in absorption at different wavelengths (350 nm to 575 nm) by subtracting the Absolute Absorption values of test (microsomal suspension with pesticides in ST buffer pH 7.4) from those of the control (microsomal suspension without pesticide in ST buffer, pH 7.4) values.

The Absorption Difference Spectra of microsomes with pesticides were classified into three types (Table 1). This modified classification is based on that used by Hodgson and Kulkarni (1974).

3.11 Inhibition of Hydrolytic Enzymes by Pesticides

3.11.1 Trypsin

3.11.1.1 Reagents: Suspended casein (50 mg, SRL, Bombay, India) in 1 ml Tris-HCl buffer,pH 9.2 and dissolved with the help of magnetic stirrer for 2 h. After centrifugation at 3000 rpm for 10 min, the dissolved casein was removed and its casein content estimated by the method of Lowry *et al.* (1951). It was 30 to 40 mg ml^{-1} at different times. This was used as substrate for proteases. Pesticides were used to study inhibition of trypsin (SRL, Bombay, 1 mg =2 units, 10 mg trypsin ml^{-1}) in PBS,pH 7.4.

3.11.1.2 Method: Added 100 µl native and inactivated (heated for 20 min boiling water bath with glass ball condensers) solution of commercial trypsin in the test and control tubes, respectively. For trypsin inhibition studies with pesticides, added 10-100 µl (providing 50 to 250 µg or 0.13 µM - 1.12 µM a.i.) of different pesticides dissolved in 1% DOC in Tris-HCl buffer, pH 8.0 and added 0.1 ml (=3 to 4 mg) casein to each tube. Made up the final volume to 1 ml with Tris-HCl buffer pH 8.5. Vortexed and immediately added 1 ml 15% TCA to the control tubes (but not to the tubes with test samples) before addition of casein as substrate. Incubated at 37°C for 2 h with shaking in a metabolic shaking water bath (Yorco, New Delhi, India). Reactions were stopped by adding 1 ml 15% TCA to all the tubes with test samples and vortexed. Refrigerated at 4°C for 2 h, centrifuged at 3000 rpm for 5 min. Aliquotes of 50 to 100 µl of supernatant were taken and used for estimation (Lowry *et al.*, 1951) of partially hydrolysed proteins. Specific activity of trypsin was expressed as mg casein hydrolysed h^{-1} mg^{-1} trypsin.

The per cent inhibition of trypsin was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Specific activity without pesticide} - \text{Specific activity with pesticide}}{\text{Specific activity without pesticide}} \times 100$$

3.11.2 Pepsin

3.11.2.1 Reagents: Added 1.5 ml 0.3N HCl, to 8 ml 2.5% BSA in 0.3N HCl, warmed at 37°C for 15 min, made up the volume to 10 ml with 0.3N HCl and used as substrate for pepsin. The solution of commercial pepsin (3000 units mg^{-1} extrapure, SRL, Bombay, India) containing 10 mg pepsin ml^{-1} in 0.01N HCl was used for inhibition by the pesticides.

3.11.2.2 Method: Added 100 μl native and inactivated (heated for 20 min in boiling water bath with glass ball condensers) solution of commercial pepsin in the test and control tubes, respectively. For pepsin inhibition studies added 10-100 μl (providing 50-250 μg or 0.13 μM - 1.12 μM a.i.) of different pesticide dissolved in 1% DOC in Tris-HCl buffer, pH 8.0 and added 0.1 ml (=2mg) BSA to each tube. Made up the volume to 1.0 ml with 0.01N HCl, vortexed and immediately added 1.0 ml 15% TCA to the control tubes. Incubated for 2 h at 37°C with shaking in a metabolic shaking water bath (Yorco, New Delhi, India). To stop the reaction, added 1.0 ml 15% TCA to all the test samples, vortexed refrigerated at 4°C for 1 h and centrifuged at 3000 rpm for 5 min. The aliquotes of 50 to 100 μl of supernatant were used for estimation (Lowry *et al.*, 1951) of partially hydrolysed proteins. Specific activity of pepsin was expressed as mg BSA hydrolysed h^{-1} mg^{-1} pepsin. The per cent inhibition of pepsin was calculated as for trypsin.

3.11.3 Microsomal Esterases (Yu *et al.*, 1984)

3.11.3.1 Reagents: The 0.25 mM solution of 1-Naphthyl acetate (LOBA Chemie, Bombay, India) (1.4 mg 1-Naphthyl acetate ml^{-1} acetone) was used as substrate. The Fast Blue B Dye solution (0.3%) was prepared in 3.5% aqueous sodium dodecyl sulfate (SDS). The microsomal suspensions prepared from liver, spleen brain, kidneys and testes and calcium chloride precipitable proteins from abomasal and duodenal mucosal cells of goats were used as source of esterases. The pesticides (Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos) dissolved in 1.0% DOC in Tris-HCl buffer, pH 8.0 were used as inhibitors of esterases.

3.11.3.2 Method: To 50 µl microsomal suspension added 10 µl to 100 µl solution of pesticides providing 50 to 250 µg or 0.13 µM to 1.12 µM of a.i.). After incubation for 15 min at 37°C, added 100 µl of 0.25 mM 1-Naphthyl acetate as a substrate and made up the final volume to 3 ml with PBS, pH 7.4. Control tubes (without pesticides) contained 50 µl of microsomal suspension, 100 µl of 0.25 mM 1-Naphthyl acetate and were made up to 3 ml with PBS, pH 7.4. All the tubes were then incubated for 1 h at 37°C. The reaction was stopped and colour was developed by adding 500 µl of 0.3% Fast Blue B Dye solution, vortexed and kept the tubes for 20 min at room temperature. Read the absorbance at 605 nm using Spectronic-20 (Bausch and Lomb, USA) against reagent blank containing 100 µl substrate solution, 2.9 ml PBS, pH 7.4 and 500 µl 0.3% Fast Blue-B Dye solution. At each step of addition the samples were vortexed. The specific activity of microsomal esterases was expressed as µmoles

product formed h^{-1} mg^{-1} microsomal proteins and the per cent inhibition of microsomal esterases was calculated as for trypsin.

All the analyses during this study were carried out in duplicate and the mean of concordant values have been documented.

CHAPTER IV

RESULTS AND DISCUSSION

The purity, composition, extraction, estimation of commercial preparations of pesticides and their residues; binding of the pesticides/their constituents to cell organelles and biomolecules; and the effects of such binding on the possible toxicity/effects of Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos on enzymes of microsome from different tissues of goats was investigated.

4.1 Characterization of Active Ingredients (a.i.) of Organophosphorus Pesticides

4.1.1 Fractionation, Detection and Quantitation of a.i.

The commercial organophosphorus pesticides and their acetone extracts fractionated by TLC and visualized with iodine vapour, Lowry reagent and cupric acetate-phosphoric acid reagent showed similar pattern (Fig.7). All the components of the eight pesticides showed dark yellow colour with iodine vapour except for Methyl parathion in which a component (Rf value 0.40) forming 15.8% showed mustard yellow colour; in Quinalphos a component (Rf value 0.75) forming 20.5% showed blue-green diffused colour; and in Dimethoate three components (Rf values 0.75, 0.51 and 0.36) forming 16.7%, 14.3% and 14.3% respectively, showed pink colour which gradually changed to

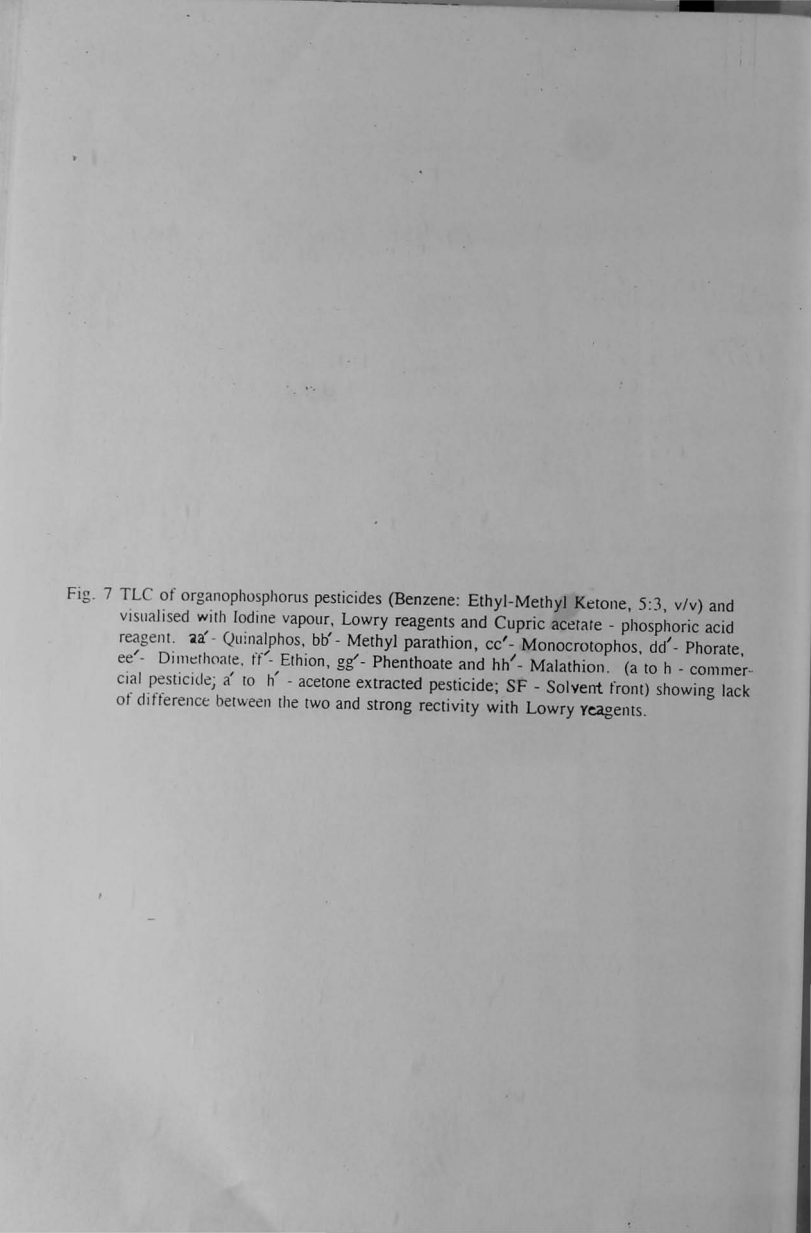
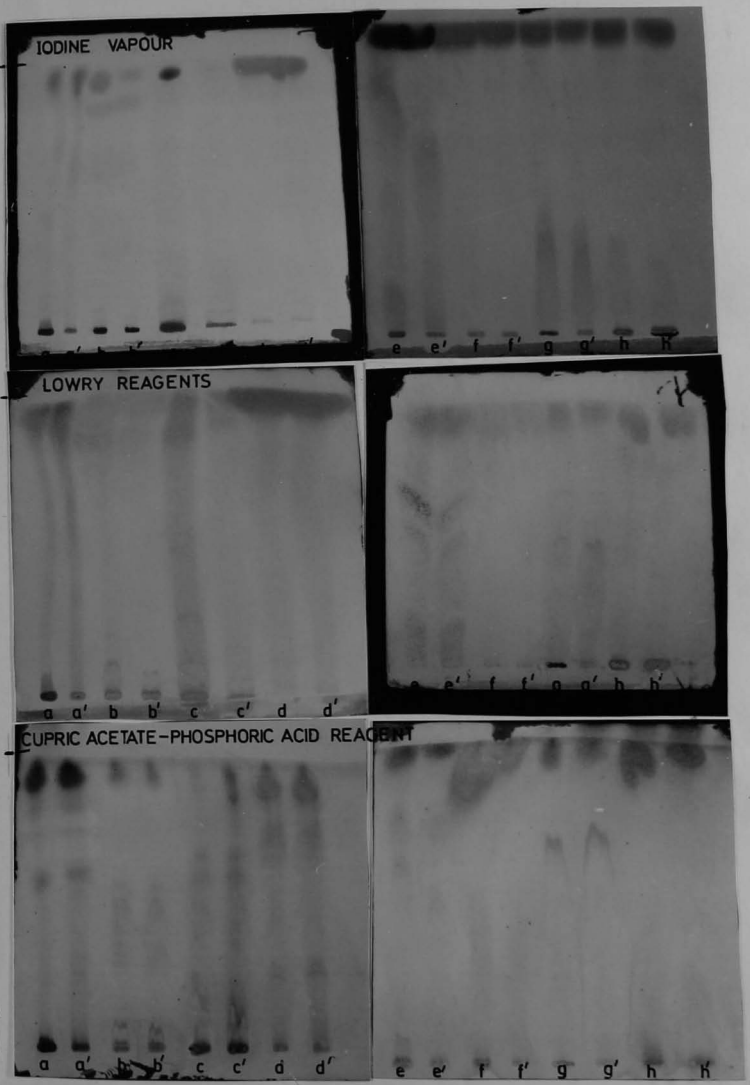


Fig. 7 TLC of organophosphorus pesticides (Benzene: Ethyl-Methyl Ketone, 5:3, v/v) and visualised with Iodine vapour, Lowry reagents and Cupric acetate - phosphoric acid reagent. aa′- Quinalphos, bb′- Methyl parathion, cc′- Monocrotophos, dd′- Phorate, ee′- Dimethoate, ff′- Ethion, gg′- Phenthoate and hh′- Malathion. (a to h - commercial pesticide; a′ to h′ - acetone extracted pesticide; SF - Solvent front) showing lack of difference between the two and strong rectivity with Lowry reagents.


IODINE VAPOUR
e e' f f' g g' h h'
LOWRY REAGENTS
a a' b b' c c' d d'
e e' f f' g g' h h'
CUPRIC ACETATE-PHOSPHORIC ACID REAGENT
a a' b b' c c' d d'
e e' f f' g g' h h'

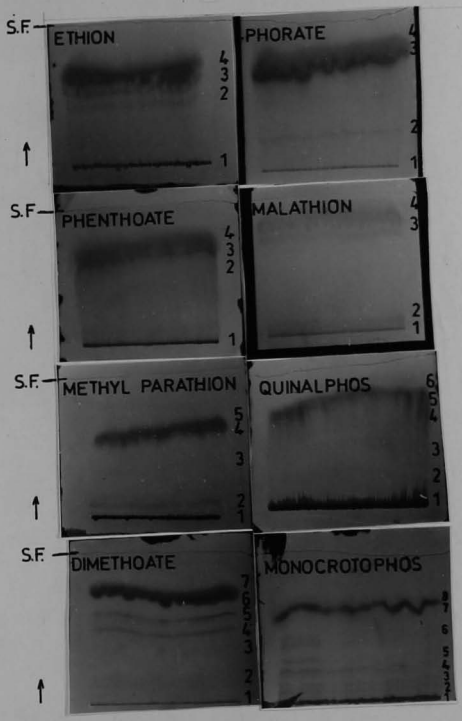


Fig. 8 TLC of organophosphorus pesticides (Benzene: Ethyl - Methyl Ketone, 5:3, v/v) showing 4 to 8 components as visualised with Iodine vapour; SF - Solvent front.

greyish brown (Fig.8). Instead of yellow colour with iodine vapour, three components of Dimethoate (Rf value 0.75, 0.51 and 0.36) and one each of Monocrotophos (Rf value 0.65) and Ethion (Rf value 0.67) showed grey brown, brown and light brown colour (Fig.8). Dimethoate, Monocrotophos and Ethion contain carbomyl methyl phosphorodithioate group, Methyl carbomyl vinyl phosphate group and Methylene di(phosphorodithioate) respectively, which are not present in other pesticides and may be responsible for this colour. The intensity of browning was higher and may be due to methyl carbomyl group (Martin, 1968).

The components of all the eight pesticides, except for Methyl parathion and Quinalphos showed blue colour with Lowry reagent. A component of Methyl parathion (Rf value 0.81) remained yellow and that of Quinalphos remained blue green (Rf value 0.93) in colour (Fig.7). Except for Methyl parathion and Quinalphos, all the components of remaining six pesticides showed black colour with cupric acetate - phosphoric acid reagent. A component (Rf value 0.81) of Methyl parathion did not show black colour with cupric acetate - phosphoric acid reagent whereas all the components except one component (Rf value 0.93, black) of Quinalphos showed brown colour (Fig.7). The mobility of the solvent system (Benzene: Ethyl methyl ketone, 5:3, v/v) during June-July months (at temperature 35.5°C) was higher compared to that during December-January months (at temperature 19.6°C). This temperature record for the year 1998 was obtained from the Meteorological Department, PAU, Ludhiana. Earlier Bakshi (1996) from this laboratory reported that Lowry reagents used for estimation of proteins (Lowry et

al., 1951) give blue colour with organophosphorus pesticides. Major component in all these pesticides were of highest Rf values.

Gravimetrically the per cent recovery of pooled acetone soluble components of Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos obtained after fractionation by TLC and elution with acetone were 74.0, 88.0, 82.0, 78.0, 88.0, 88.0, 84.0 and 82.0 per cent, respectively. The recovery as determined from the phosphorus content of the fractions were 50.5, 58.6, 61.2, 81.9, 53.6, 73.7, 63.3 and 63.9 per cent, respectively. Except for Malathion the per cent recovery of the pesticides was higher by gravimetric method. These differences may be due to the presence of acetone soluble, base components lacking phosphorus.

All the components of the eight pesticides (Table 5) were phosphorus containing compounds. The results indicate that commercially available preparations of these 8 pesticides are made up of 4 to 8 phosphorus containing components.

4.1.2 Pesticide Components not Bound by Trypsin (Fig.9)

All the components of Quinalphos were completely bound to trypsin except those with Rf values of 0.03 and 0.93 were bound partially to trypsin. All the components of Methyl parathion were bound to trypsin except those with Rf values of 0.91, 0.81 and 0.03 which were bound partially. All the components of Monocrotophos were completely bound to trypsin except those with Rf values of 0.94 and 0.03. Except one component of Rf value 0.93 all the components of Phorate were

Table 5. Proportion of different components of organophosphorus pesticides, fractionated by TLC and determined gravimetrically

Component No.	Ethion	Phorate	Phenthoate	Malathion	Methyl-parathion	Quinalphos	Dimethoate	Monocrotophos
1	27.0(.01)	4.5(.01)	53.6(.02)	15.4(.02)	11.4(.07)	20.5(.06)	11.9(.05)	19.5(.02)
2	13.5(.41)	11.4(.12)	4.9(.60)	12.8(.06)	22.7(.11)	13.6(.24)	16.7(.145)	9.8(.07)
3	5.5(.54)	11.4(.52)	29.3(.70)	12.8(.71)	11.4(.13)	13.6(.43)	**14.3**[c](.36)	7.3(.14)
4	**54.0**[e](.67)	72.7(.77)	12.2(.81)	58.9(.85)	**15.8**[a](.40)	9.0(.57)	**14.3**[c](.51)	4.9(.24)
5					38.7(.60)	22.8(.66)	21.4(.59)	7.3(.31)
6						**20.5**[b](.75)	**16.7**[c](.75)	12.2(.41)
7							4.7(.81)	14.6(.57)
8								**24.4**[d](.65)
Recovery mg	37	44	41	39	44	44	42	41
Per cent	74	88	82	78	88	88	84	82

50 mg of each pesticide was fractionated. The Rf values are indicated in the parentheses.
a, mustard yellow colour; b, blue-green colour diffused at junction; c, pink to greyish brown;
d, brown; e, light brown; in presence of iodine vapour

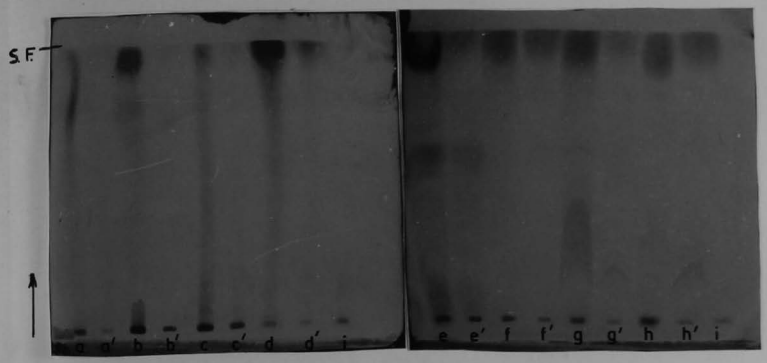


Fig. 9 TLC of organophosphorus pesticide components not bound to Trypsin. aa′- Quinalphos. bb′- Methyl parathion, cc′- Monocrotophos, dd′- Phorate, ee′- Dimethoate, ff′- Ethion, gg′- Phenthoate, hh′- Malathion and i - 1% DOC in Tris-HCl buffer, pH 8.0 (a to h - acetone extracted pesticide; a′ to h′ - not bound pesticide; SF - Solvent front).

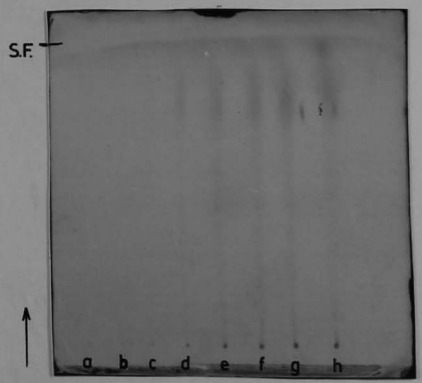


Fig. 10 TLC pattern of Quinalphos (Benzene: Ethyl-Methyl Ketone, 5:3, v/v) visualised with Lowry reagents to determine the lower limits of detection. a to h are : 3.12, 6.25, 12.5, 25, 50, 100, 200 and 400 ug of Quinalphos, respectively. The component with 0.93 Rf value is detectable upto 624 ppb levels.; SF-Solvent front.

completely bound to trypsin. The components of Dimethoate with Rf values of 0.97, 0.65, 0.15 and 0.03 were partially bound to trypsin. All the components of Ethion except those with Rf values of 0.03 and 0.97 were bound to trypsin and those of Phenthoate (except one with Rf value of 0.67) were partially bound to trypsin. All the components of Malathion except one were partially bound to trypsin and one with Rf value of 0.28 did not bind to trypsin.

The a.i. of Methyl parathion, Quinalphos and Dimethoate gave characteristic colours after visualisation with iodine vapour and Lowry reagents. These were selected for determining the lower limits of detection of their residues and to devise method(s) for their quantitation. The results indicate that a component of Quinalphos with Rf value of 0.93 (forming 20.5%) could be detected upto 624 ppb (Fig.10). A component of Methyl parathion with Rf value 0.81 (forming 15.8%) could be detected upto 938 ppb. A component of Dimethoate with Rf value 0.93 (forming 16.7%) could be detected upto 521 ppb.

4.1.3 Absorption Spectra of a.i. of Pesticides Complexed with Lowry Reagents (Fig.12)

All the pesticides complexed with Lowry reagents gave maximum absorption at 660 nm. Dimethoate, Monocrotophos, Phorate, Malathion, Phenthoate, Quinalphos, Methyl parathion and Ethion absorbed in decreasing order at 660 nm. Second peak of lower intensity was observed at 520 nm with all the pesticides except Quinalphos and Methyl parathion which showed maximum absorption at 380 nm and 410 nm respectively. Methyl parathion gave yellow colour with Lowry reagent which was not

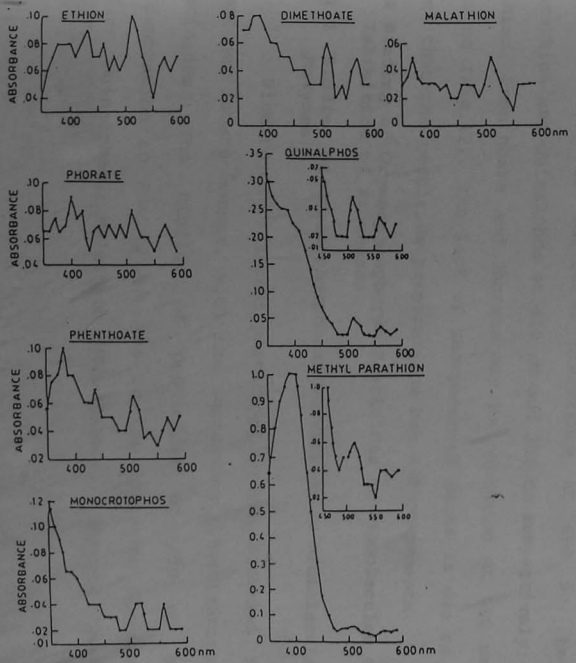


FIG.11. ABSOLUTE ABSORPTION SPECTRA OF ORGANOPHOSPHORUS PESTICIDES

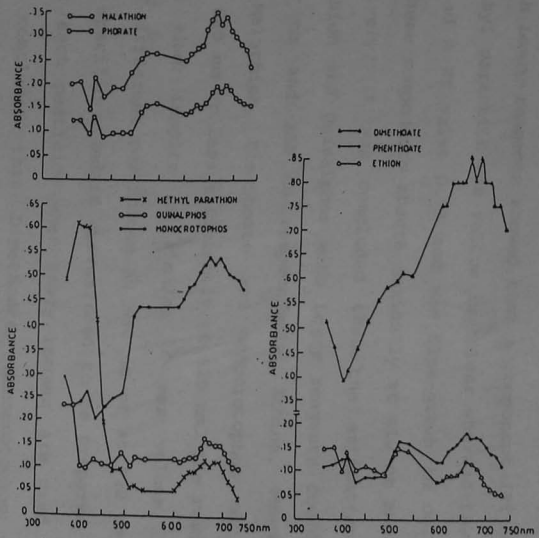


FIG.12 ABSOLUTE ABSORPTION SPECTRA OF ORGANOPHOSPHORUS PESTICIDES (250µg a.i.) REACTED WITH THE LOWRY REAGENTS AS USED FOR PROTEINS

observed with other pesticides. Its fractionation on TLC and staining with Lowry reagents showed that a component (Rf value 0.40) of Methyl parathion was yellow in colour whereas that of Quinalphos had a Rf value 0.75 and was blue-green in colour. Apparently these components absorb maximally at 410 nm and 380 nm, respectively. It was concluded that the estimation of Methyl parathion and Quinalphos with Lowry reagents could be done at 410 nm and 380 nm respectively. Ethion, Phorate, Phenthoate, Malathion, Dimethoate and Monocrotophos may be estimated at 660 nm or less efficiently at 520 nm. The results also suggest that inspite of similar λ max values, the absorbance of different pesticides at 660 nm or at 520 nm is variable. So specific standard curves need to be prepared for estimation of each pesticide when Lowry reagent are used. It was further concluded that Dimethoate, Monocrotophos and Malathion may be estimated preferably at 660 nm; Methyl parathion and Quinalphos at 410 nm and 380 nm respectively; and Ethion, Phenthoate and Phorate may be estimated at 660 nm or 520 nm (Fig.12). The lack of sharpness of peaks is due to the fact that the pesticide preparations has 4-8 components.

4.2 Binding of Organophosphorus Pesticides to Biomolecules and Inhibition of Proteases

4.2.1 Binding of Organophosphorus Pesticides to Biomolecules/ Enzymes

Binding of different pesticides to BSA increased, in a disproportionate manner, with the increasing concentration of pesticides. The binding of pesticides at 250 µg a.i. concentration to BSA (2 mg) was in the following decreasing order: Quinalphos, Malathion (Phenthoate), Phorate (Methyl

parathion), Ethion (Monocroto- phos) and Dimethoate (Table 6, Fig.13). The binding of various pesticides at 50 μg a.i. to BSA (2 mg) was in the following decreasing order: Quinalphos, Phenthoate (Methyl parathion), Monocrotophos, Ethion (Phorate and Malathion) and Dimethoate. Since, out of 250 μg pesticide a.i., 80% to 100% was bound to pepsin, so 50 μg or less of the pesticide is left for binding to the BSA. At 50 μg concentration the binding of these pesticides to BSA is 16% to 71%. Therefore, observed inhibition of pepsin is mainly due to their binding to the pepsin. The binding of pesticides (with low LD_{50}) to BSA is relatively low, so their transport by serum albumen will also be low after absorption. Per cent binding to casein (2.3 mg) by 250 μg a.i. pesticides was in the following decreasing order: Phenthoate, Ethion, Quinalphos (Methyl parathion), Dimethoate (Malathion), Phorate and Monocrotophos. At concentration of 50 μg a.i. pesticide and 2.3 mg casein, binding of Phenthoate, Dimethoate and Ethion was maximum, whereas, binding of Monocrotophos, Phorate and Quinalphos was lower. Phenthoate, Ethion, Methyl parathion, Monocrotophos showed gradual increase in per cent binding to casein (Table 6, Fig.13). Out of 250 μg a.i. of the various pesticides and 1 mg trypsin, 53% to 86% (except Malathion) bind to trypsin and 30% and 47% (75 and 125 μg a.i.) of the pesticides (except Malathion) remain available for binding to casein. The binding of various pesticides at these concentrations to casein varies between 7% to 50% and 10% to 58%, respectively. Therefore, casein as substrate may sequester the pesticides. Casein (a phosphoprotein) and other proteins may, therefore, decrease

Table 6. Binding of organophosphorus pesticides to bovine serum albumen (BSA) and casein

Pesticide	Pesticide (μg a.i.)					Pesticide (μg a.i.)				
	50	100	150	200	250	50	100	150	200	250
	% binding to BSA					% binding to Casein				
Ethion	33.3	41.6	44.4	58.3	60.0	33.3	37.5	41.6	75.0	75.0
Phorate	33.3	50.0	55.0	58.3	66.6	16.6	16.6	37.5	38.8	40.0
Phenthoate	50.0	62.5	75.0	75.0	80.0	50.0	58.3	72.2	91.6	100.0
Malathion	33.3	50.0	66.7	75.0	80.0	31.3	37.5	45.8	51.5	55.0
Methyl parathion	50.0	50.0	50.0	62.5	65.0	25.0	50.0	50.0	56.2	60.0
Quinalphos	71.0	78.5	80.9	82.1	88.5	18.2	18.2	18.2	60.2	63.6
Dimethoate	16.7	41.6	44.4	50.0	56.6	43.2	46.6	47.7	53.3	56.5
Monocrotophos	40.0	50.0	53.3	57.5	60.0	7.1	10.7	11.9	30.3	34.3

Incubation conditions: 2 mg BSA and 2.3 mg casein in appropriate buffer were mixed with 10 μl to 100 μl pesticide solution (providing 50 μg to 250 μg a.i.) and made up the final volume to 0.2 ml with 1% DOC in Tris-HCl buffer, pH 8.0, incubated for 15 min at 37°C; extracted and estimated the unbound pesticides

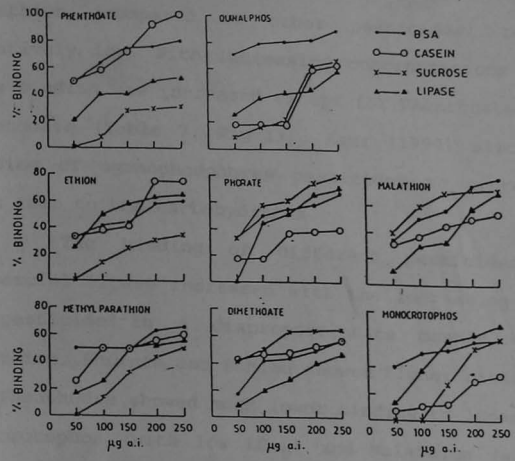


FIG.13. EFFECT OF DIFFERENT CONCENTRATIONS (µg a.i.) OF ORGANOPHOSPHORUS PESTICIDES ON PERCENT BINDING TO BSA, CASEIN, LIPASE AND SUCROSE

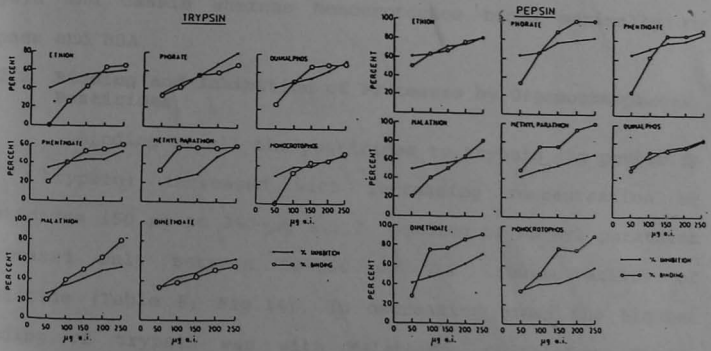


FIG.14. EFFECT OF DIFFERENT CONCENTRATIONS (µg a.i.) OF ORGANOPHOSPHORUS PESTICIDES ON PERCENT INHIBITION/ BINDING TO PURIFIED COMMERCIAL TRYPSIN AND PEPSIN

the availability of pesticide for absorption as well as for inhibition of digestive enzymes.

The binding, of Ethion, Phenthoate and Methyl parathion (compared to other pesticides) to sucrose was relatively low. With increasing concentrations of pesticides, this binding was increased except for Phenthoate, Malathion and Dimethoate (Table 7, Fig.13). Kaur (1996) also reported that binding of oganophosphorus pesticides to proteins is higher than that to the carbohydrates.

The binding of different pesticides to purified commercial lipase increased with the increasing concentrations of pesticide in a disproportionate manner. Monocrotophos, Malathion, Phorate and Ethion showed higher binding. Dimethoate and Phenthoate showed much lower binding to lipase. Phorate and Monocrotophos (with low LD_{50}) and Malathion (with high LD_{50}) showed higher binding to commercial lipase (Table 7, Fig.13). Kaur (1996) reported that Methyl parathion binds maximally to pepsin and casein whereas Monocrotophos binds maximally to lipase and BSA.

4.2.2 Binding and Inhibition of Proteases by Organophosphorus Pesticides

Binding of all the pesticides to trypsin (μg pesticide mg^{-1} trypsin) increased with increasing concentration of pesticides (50 μg to 250 μg a.i.). Binding of Methyl parathion increased only between 50-100 μg a.i. concentrations of pesticide (Table 8, Fig.14). In decreasing order the highest binding of trypsin was with Malathion, Quinalphos, Ethion (Phorate), Phenthoate (Methyl parathion), Dimethoate and Monocrotophos. The per cent inhibition of trypsin increased

Table 7. Binding of organophosphorus pesticides to lipase and sucrose

Pesticide	Pesticide (μg a.i.)					Pesticide (μg a.i.)				
	50	100	150	200	250	50	100	150	200	250
	% binding to Lipase					% binding to Sucrose				
Ethion	25.0	50.0	58.3	62.5	65.0	-	12.5	25.0	31.3	35.0
Phorate	-	45.5	50.0	65.5	70.0	33.3	58.3	61.1	75.0	80.0
Phenthoate	20.0	40.0	40.0	50.0	52.0	-	8.3	27.7	29.2	30.0
Malathion	10.0	30.0	33.3	57.5	70.0	40.0	60.0	70.0	70.0	72.0
Methyl parathion	16.7	25.0	50.0	54.0	56.0	-	12.5	33.3	43.8	50.0
Quinalphos	25.0	37.5	41.6	43.7	60.0	8.3	16.6	22.2	62.5	76.6
Dimethoate	8.3	20.8	27.7	39.5	48.3	41.6	50.0	62.5	63.5	65.0
Monocrotophos	12.5	37.5	58.3	62.5	70.0	-	-	27.7	54.2	60.0

Incubation conditions: 4.5 mg commercial lipase and 0.34 mg sucrose in appropriate buffer were mixed with 10 μl to 100 μl pesticide solution (providing 50 μg to 250 μg a.i.) and made up the final volume to 0.2 ml with 1% DOC in Tris-HCl buffer, pH 8.0, incubated for 15 min at 37°C; extracted and estimated the unbound pesticides

Table 8. Binding of organophosphorus pesticides to purified, commercial proteases

Pesticide	Pesticide (μg a.i.)					Pesticide (μg a.i.)				
	50	100	150	200	250	50	100	150	200	250
	% binding to Trypsin					% binding to Pepsin				
Ethion	-	25.0	41.6	62.5	65.0	50.0	62.5	66.6	75.0	80.0
Phorate	33.3	41.6	55.5	58.3	66.7	33.3	66.7	88.9	100.0	100.0
Phenthoate	20.0	40.0	53.3	55.0	60.0	25.0	62.5	84.6	85.0	90.4
Malathion	40.0	45.0	46.6	60.0	86.0	20.0	40.0	50.0	62.5	80.0
Methyl parathion	33.3	58.3	58.3	58.3	60.0	50.0	75.0	75.0	93.8	100.0
Quinalphos	25.0	50.0	66.7	68.7	70.0	50.0	68.8	70.8	75.0	82.5
Dimethoate	33.3	37.5	44.4	52.0	55.0	28.0	75.0	76.2	85.7	91.4
Monocrotophos	-	33.3	44.0	45.8	53.3	33.3	50.0	77.0	75.0	90.4

Incubation conditions: 1 mg purified, commercial protease in appropriate buffer was mixed with 10 μl to 100 μl pesticide solution (providing 50 μg to 250 μg a.i.) and made up the final volume to 0.2 ml with 1% DOC in Tris-HCl buffer, pH 8.0 incubated for 15 min at 37°C; extracted and estimated the unbound pesticides

with the increasing proportion of pesticides except with Ethion and Phenthoate. With Phenthoate and Ethion, inhibition did not increase at 100-200 μg a.i., respectively (Table 9, Fig.14). In decreasing order the highest inhibition of trypsin was achieved with 250 μg a.i. of Phorate, Quinalphos, Dimethoate, Ethion, Methyl parathion, Malathion, Phenthoate and Monocrotophos. With 50 μg a.i. concentration of pesticides the lowest inhibition was with Methyl parathion and highest with Quinalphos; inhibition by Ethion, Phorate, Phenthoate and Dimethoate were almost similar and same was true for Malathion and Monocrotophos. The results (Table 9, Fig.14) suggest that the inhibition of trypsin by organophosphorus pesticides is disproportionate to the per cent pesticide bound to trypsin. With increasing concentration of Malathion, there is higher increase in binding, compared to the increase in inhibition of trypsin. Same is true to varying degrees for Phenthoate, Methyl parathion, Quinalphos and Ethion. Such results suggest that binding of these pesticides to trypsin takes place also at sites other than the active centre of trypsin and results in disproportionate inhibition due to sequestering of these pesticides. The increase in binding with increasing concentrations of Monocrotophos, Dimethoate and Phorate, was associated with corresponding or slightly higher increase in inhibition of trypsin, suggesting that these pesticides bound more to catalytic sites of trypsin than to non-catalytic ones leading to higher inhibition by these three pesticides as their concentration is increased.

The higher inhibition of trypsin by Quinalphos may

Table 9. *In vitro* inhibition of purified, commercial trypsin by organophosphorus pesticides

Pesticide (μg a.i)	Ethion	Phorate	Phenthoate	Malathion	Methyl-parathion	Quinalphos	Dimethoate	Mono-crotophos
				Sp.Act. (mg casein hydrolysed h^{-1} mg^{-1} trypsin)				
-	.625	.625	.625	.625	.625	.625	.625	.625
50	.375	.398	.406	.461	.507	.352	.422	.445
100	.320	.336	.367	.406	.469	.313	.352	.383
150	.281	.281	.352	.367	.445	.289	.328	.375
200	.266	.203	.352	.313	.320	.234	.258	.328
250	.258	.125	.297	.295	.258	.164	.234	.305
				Per cent Inhibition				
50	40.0	36.3	35.0	26.2	18.9	43.7	32.5	28.8
100	48.8	46.2	41.3	35.0	25.0	49.9	43.7	38.7
150	55.0	55.0	43.7	41.3	28.8	53.8	47.5	40.0
200	57.4	67.5	43.7	49.9	48.8	62.6	58.7	47.5
250	58.7	80.0	52.5	52.8	58.7	73.8	62.6	51.2

Incubation conditions: Trypsin activity was determined in Tris-HCl buffer (pH 8.5) at 37°C for 2 h, with 1 mg purified trypsin and 3 mg to 4 mg casein in 1 ml final volume of reaction mixture.

also be due to stability of the latter (Martin, 1968) in the alkaline medium, thus making it available for binding to trypsin. The highest binding but relatively lower inhibition of trypsin by Malathion may be due to its instability at alkaline pH as well as due to binding at non-catalytic sites of trypsin thus leading to its sequestering under these conditions. Martin (1968) reported that Malathion is unstable at pH >7.0 and <5.0 and has high oral LD_{50} for rats (2800 kg^{-1} b.w.). Therefore, under *in vivo* conditions Malathion is likely to be degraded and sequestered in the digestive tract of mammals. This may also decrease the availability of Malathion for absorption and subsequent action in the internal organs. Aldridge (1953) reported that the tissues which show uninhibitable esterase activity in the presence of pesticides, may be capable of hydrolysing ester linkages of the pesticide. Malathion (Fig.5) has two carboxyl ester linkages which may be hydrolysed by such esterases before and after absorption from gastrointestinal tract. These esterases may also detoxify the absorbed Malathion and thus increase its tolerance and oral LD_{50} dose. Sun *et al.* (1992) and Brealey *et al.*(1980) reported that carboxyl-esterase-A degrades Malathion and its activity is low in the plasma of birds as compared to those of mammals. However, these authors have not checked the degradation and sequestering of Malathion in the digestive tract of birds, which could modify the amount of absorbed Malathion. Verma and Ahuja (1998) observed that oral administration of low doses of Methyl parathion for 49 days to male rats increases the esterase activity in testicular fluids but decreases that in plasma and

also be due to stability of the latter (Martin, 1968) in the alkaline medium, thus making it available for binding to trypsin. The highest binding but relatively lower inhibition of trypsin by Malathion may be due to its instability at alkaline pH as well as due to binding at non-catalytic sites of trypsin thus leading to its sequestering under these conditions. Martin (1968) reported that Malathion is unstable at pH >7.0 and <5.0 and has high oral LD_{50} for rats (2800 kg^{-1} b.w.). Therefore, under *in vivo* conditions Malathion is likely to be degraded and sequestered in the digestive tract of mammals. This may also decrease the availability of Malathion for absorption and subsequent action in the internal organs. Aldridge (1953) reported that the tissues which show uninhibitable esterase activity in the presence of pesticides, may be capable of hydrolysing ester linkages of the pesticide. Malathion (Fig.5) has two carboxyl ester linkages which may be hydrolysed by such esterases before and after absorption from gastrointestinal tract. These esterases may also detoxify the absorbed Malathion and thus increase its tolerance and oral LD_{50} dose. Sun *et al.* (1992) and Brealey *et al.*(1980) reported that carboxyl-esterase-A degrades Malathion and its activity is low in the plasma of birds as compared to those of mammals. However, these authors have not checked the degradation and sequestering of Malathion in the digestive tract of birds, which could modify the amount of absorbed Malathion. Verma and Ahuja (1998) observed that oral administration of low doses of Methyl parathion for 49 days to male rats increases the esterase activity in testicular fluids but decreases that in plasma and

testicular tissue. This suggested that under *in vivo* conditions low doses of Methyl parathion inhibit and induce the testicular esterase and also bring about necrotic changes in the testes. The decrease in serum esterase activity suggested its inhibition and possibly due to lack of induction in hepatic and other tissues.

Binding of various pesticides to pepsin was relatively higher than that of trypsin (Table 8, Fig.14). The per cent binding of various pesticides to each mg of commercial pepsin increases as the concentration of the pesticide is increased from 50 to 250 µg a.i. In decreasing order the highest binding to pepsin was with Phorate, Methyl parathion, Dimethoate, Phenthoate (Monocrotophos), Quinalphos and Ethion (Malathion). The per cent inhibition of pepsin increased with the increasing proportion of pesticides. In decreasing order the highest inhibition of pepsin was achieved with 250 µg a.i. of Phenthoate, Quinalphos, Phorate, Ethion, Dimethoate, Methyl-parathion, Malathion and Monocrotophos (Table 10, Fig.14). With increasing concentrations of Monocrotophos, Methyl parathion, Dimethoate, Phorate and Phenthoate, the binding of pesticides to pepsin increases in a disproportionate manner as compared to the increase in inhibition of pepsin. Per cent binding of Quinalphos and Ethion to pepsin and inhibition of pepsin correspondingly increase with increasing concentration of pesticides.

In case of Malathion the inhibition of pepsin is highly disproportionate to the binding. The inhibition is higher and almost constant with lower concentrations and the

Table 10. *In vitro* inhibition of purified, commercial pepsin by organophosphorus pesticides

Pesticide (μg a.i)	Ethion	Phorate	Phenthoate	Malathion	Methyl-parathion	Quinalphos	Dimethoate	Mono-crotopho
			Sp.Act.(mg casein hydrolysed h^{-1} mg^{-1} trypsin)					
-	.836	.836	.836	.836	.836	.836	.836	.836
50	.328	.313	.289	.430	.484	.383	.492	.563
100	.309	.285	.242	.383	.430	.297	.445	.492
150	.242	.195	.184	.375	.383	.227	.391	.484
200	.215	.172	.176	.320	.328	.203	.328	.430
250	.164	.152	.113	.305	.281	.141	.234	.352
			Per cent Inhibition (%)					
50	60.8	62.6	65.4	48.6	42.1	54.2	41.2	32.7
100	63.0	65.9	71.1	54.2	48.6	64.5	46.8	41.2
150	71.1	76.7	78.0	55.1	54.2	72.9	53.2	42.1
200	74.3	79.4	79.0	61.7	60.8	75.7	60.8	48.6
250	80.4	81.8	86.5	63.5	66.4	83.1	72.0	57.9

Incubation conditions: Pepsin activity was determined in 0.01 N HCl at 37°C for 2 h, with 2 mg bovine serum albumin in 1 ml final volume of reaction mixture.

per cent binding is higher compared to the per cent inhibition at higher concentrations of Malathion. From the results, it may be concluded that binding of Monocrotophos, Methyl parathion, Phorate and Dimethoate to pepsin takes place at sites other than the catalytic site of pepsin.

Ethion, Phorate and Phenthoate also maximally inhibit pepsin at concentration between 50-250 μg. This may be due to the fact that these pesticides are stable at acid pH and also bind maximally to pepsin. The higher inhibition of pepsin by Quinalphos, an alkali labile pesticide, may be due to its higher binding to pepsin especially at 50 μg concentration. The lower inhibition by Malathion may be due to its poor binding to pepsin and susceptibility to hydrolysis in acidic medium. The poor inhibition of pepsin by Monocrotophos, inspite of higher binding may be due to its higher hydrolysis under acidic conditions. Same may be true for Dimethoate and Methyl-parathion. The *in vitro* inhibition of enzymes of digestive tract contents of goats and purified commercial trypsin, pepsin and amylase was increased with increasing concentration of Monocrotophos, Phenthoate and Malathion, Quinalphos, Dimethoate, Phorate, Methyl parathion. Maximal inhibition was observed with lipase (Kaur, 1996 and Bakshi, 1996). Bakshi (1996) reported that inhibition of pepsin, amylase, lipase(s) and pancreatic protease(s) (from particulate free goat duodenal contents) was more with Malathion than with Phenthoate.

4.3 Binding of Pesticides to Microsomes and Inhibition of Microsomal Esterases

4.3.1 Binding of Pesticides to Microsomes

The binding (Table 11) of Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos (250 μg a.i.) to the microsomal preparations from brain (=14.0 mg protein), testes (=9.0 mg protein) and kidneys (=10.7 mg protein) were higher than to those from liver (=25.3 mg protein) and spleen (=12.7 mg protein) of goats. Binding of various pesticides to hepatic microsomal preparations varied between 40% for Malathion and to greater than 70% for Quinalphos, Phenthoate and Phorate. The binding of various pesticides to spleen microsomes varied between 40% for Ethion and greater than 70% for Quinalphos, Dimethoate and Malathion; and intermediate values were observed for Methyl parathion, Phorate, Phenthoate and Monocrotophos. The binding of all the pesticides to brain microsomes was higher (75 t0 100%) than to those from other tissues. The binding of Phorate and Dimethoate was 100% and 80% or above in case of Quinalphos, Monocrotophos and Phenthoate. The binding of various pesticides to kidney microsomes varied between 70% to 76% for Malathion, Monocrotophos, Phenthoate and Ethion; and between 79 to 89% for Methyl parathion, Phorate, Quinalphos and Dimethoate. The binding of various pesticides to testes microsomes was 40% to 56% for Monocrotophos, Malathion and Phenthoate and between 78% to 93% for Dimethoate, Methyl parathion, Ethion, Quinalphos and Phorate. The higher binding of pesticides to microsomal preparations from brain compared to those of other tissues suggested that brain microsomal functions may be affected more

Table 11. Per cent and type of binding of organophosphorus pesticides to microsomes prepared from different tissues of goats

Pesticide	Liver	Spleen	Brain	Kidneys	Testes
		% Binding with microsomes			
Ethion	46.7	40.0	80.0	73.3	80.0
Phorate	73.3	63.3	100.0	83.3	93.3
Phenthoate	76.7	50.0	90.0	73.3	56.7
Malathion	40.0	70.0	86.7	70.0	50.0
Methyl parathion	54.2	66.7	75.0	79.2	79.2
Quinalphos	77.1	78.8	93.9	89.4	87.9
Dimethoate	65.0	70.0	100.0	88.3	78.3
Monocroto-phos	56.7	50.0	93.3	76.7	40.0
	Type of binding (as explained in Table 1)				
Ethion	II,Rev.I	I	II,Rev.I,	-	II,Rev.I,
Phorate	II,Rev.I	I	II,Rev.I,	I	II,Rev.I
Phenthoate	II,Rev.I,	I	-	I	I
Malathion	II,Rev.I	I	I	Rev.I	II,Rev.I
Methyl parathion	Rev.I	Rev.I	I	II,Rev.I	I
Quinalphos	I	I	I	I	II,Rev.I
Dimethoate	I	I	I	I	II,Rev.I
Monocroto-phos	II,Rev.I	I	I	I	II,Rev.I,

Incubation conditions: 50 µl to 100 µl pesticide solution (providing 250 µg a.i.) in 1.8 ml microsomal preparation (from 1.8g tissue) and made up the final volume to 2 ml with ST buffer, pH 7.5; incubated for 15 min at 26°C; extracted and estimated the unbound pesticide.

adversely. Kaur *et al.*(1996) reported that Malathion and Phenthoate strongly bind to microsomes of testes of rats. The binding of Monocrotophos was less than that of Malathion and Phenthoate.

The type of binding of pesticides to microsomes from different tissues of goats was deduced from the Absorption Difference Spectra (Figs.15,16, Table 12). All the pesticides except Methyl parathion (which showed Reverse Type I binding) showed Type-I binding with microsomes from splenocytes; Quinalphos and Dimethoate with microsomes from liver; Malathion and Monocrotophos with microsomes from brain; Phorate, Phenthoate, Malathion, Dimethoate and Monocrotophos with microsomes from kidney and Phenthoate and Methyl parathion with microsomes from testes (Table 11). The results suggested binding of these pesticides to a lipophillic site away from heme of the hemoproteins of microsomes. Ethion, Phorate, Phenthoate, Malathion, Methyl parathion and Monocrotophos showed Reverse Type I binding with hepatic microsomes; Ethion and Phorate with microsomes from brain; Methyl parathion and Quinalphos with microsomes from kidney; all the pesticides (except Phenthoate and Malathion) with microsomes from testes. These results indicated binding of pesticides by displacement of an endogenous substrate or binding of nucleophillic oxygen to heme iron of hemoproteins of microsomes (Table 11). Ethion and Phorate showed Type II binding with microsomes from liver, brain and testes; Phenthoate with microsomes from liver; Quinalphos with microsomes from kidney and testes; Dimethoate with microsomes from testes; Monocrotophos with microsomes from

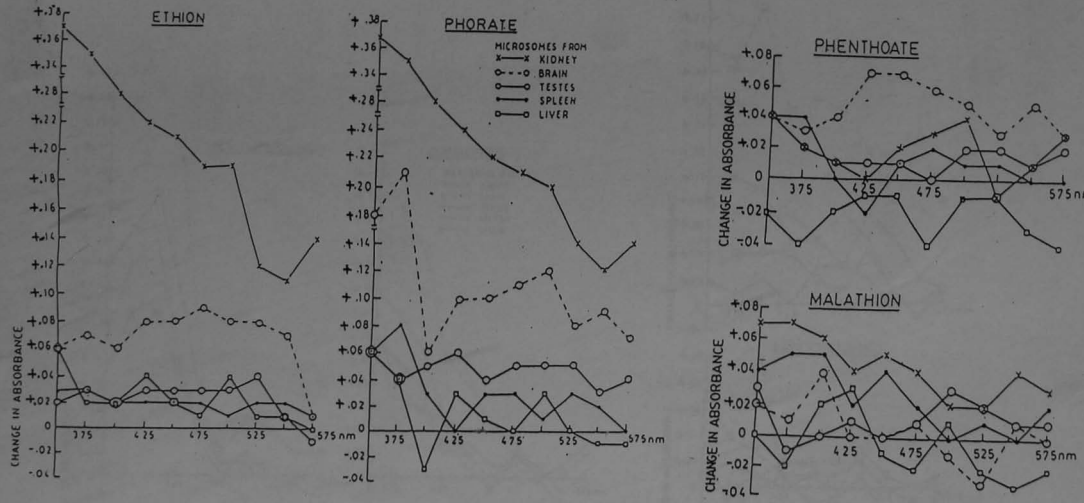


Fig. 15. ABSORPTION DIFFERENCE SPECTRA OF DIFFERENT ORGANOPHOSPHORUS PESTICIDES (250 μg a.i.) WITH MICROSOMES FROM DIFFERENT TISSUES OF GOATS.

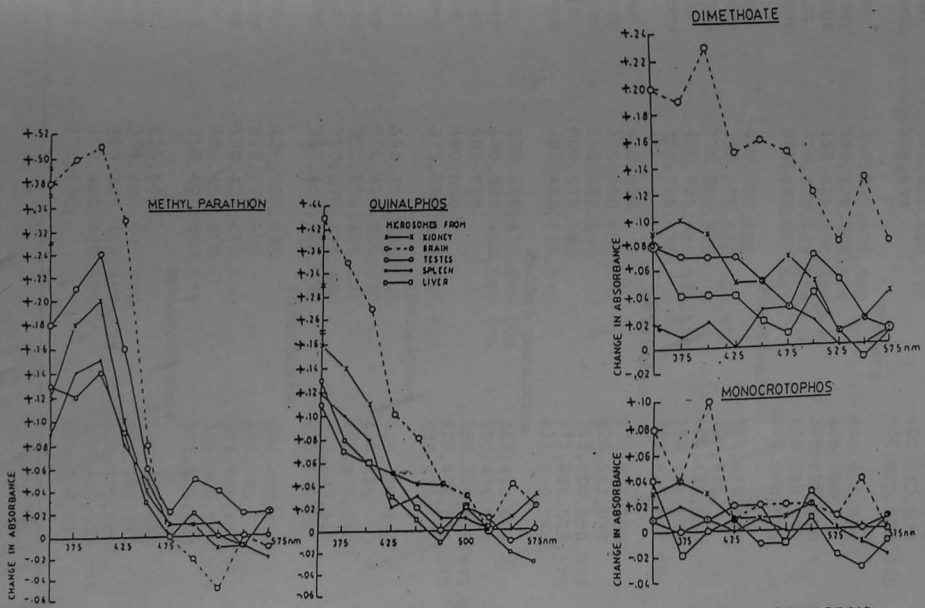


Fig. 16. ABSORPTION DIFFERENCE SPECTRA OF DIFFERENT ORGANOPHOSPHORUS PESTICIDES (250 µg a.i.) WITH MICROSOMES FROM DIFFERENT TISSUES OF GOATS.

Table 12. Absorption Difference Spectra (peaks and troughs) of microsomes from different tissues of goats in presence of 250 μg a.i. of organophosphorus pesticides

Tissue	Peaks (nm)	Troughs (nm)
	Ethion	
Liver	425,500	375,400,475,525
Kidney	350,425,450,525,575	475,550
Testes	375,425,450,475,525	400,550,575
Brain	375,425,450,475	400,512,575
Spleen	375,537	400,425,500,575
	Phorate	
Liver	350,375,425,500	400,475,550
Kidney	350,375,500,575	425,500
Testes	350,425,500,525,575	375,425,550
Brain	375,425,450,500,550	400,525,575
Spleen	375,450,475,525	425,500,575
	Phenthoate	
Liver	425,450,525	375,475,575
Kidney	350,375,450,500,575	425,525
Testes	375,500,575	400,425,550
Brain	400,425,450,500,550	375,525,575
Spleen	350,375,475	400,425,512,550
	Malathion	
Liver	425,500	375,475,550
Kidney	375,450,550	425,512,575
Testes	350,425,500	375,450,550,575
Brain	400,475,550	375,425,525,575
Spleen	387,450,525,575	425,500,550
	Methyl parathion	
Liver	350,400,425,450,500	375,475,550
Kidney	400,425,450,500,575	350,475,525
Testes	400,500	350,375,425,475,550
Brain	400,550	425,475,525,575
Spleen	375,400,425,450,525	500,575
	Quinalphos	
Liver	350,375,400,500	425,475,550
Kidney	350,425,450,575	400,525
Testes	350,375,425,500	400,475,550
Brain	350,375,475,550	425,525,575
Spleen	350,400,450,575	425,500
	Dimethoate	
Liver	425,500,575	375,475,550
Kidney	375,475,575	425,525
Testes	350,425,450,500	375,400,475,550,575
Brain	400,450,550	375,425,525,575
Spleen	350,400,450,575	375,425,525
	Monocrotophos	
Liver	425,500	375,475,550
Kidney	375,400,500,575	425,450,550
Testes	350,425,450,500,575	375,475,550
Brain	350,400,475,500	375,425,525,575
Spleen	375,450	425,500,575

liver and testes. These results suggested binding of pesticides to heme iron of hemoproteins of microsomes (Table 11). These conclusions are based on the binding characteristics of Type I, Type II and Reverse Type I ligands to microsomes reported by Hodgson and Kulkarni (1974). Since deduced binding of pesticides may modify heme of Cyt.P-450 component of microsomes of liver and testes by Type II or Reverse Type I reaction, so metabolic functions in these tissues may be affected adversely. Microsomes from spleen showed Type I binding with these pesticides, so less adverse effects can be expected in spleen due to modification of structures around the heme. Sato *et al.*(1969) reported similar finding with rabbit liver microsomes after reduction of Cyt.P-450.

The binding at the lipophillic sites and increased reduction of complexes may not only lead to sequestering and detoxification of the pesticides but may also decrease the availability of NADPH for biosynthetic purposes. Venkateswarlu *et al.*(1996a) reported that azoles (antifungal agents) show Type II binding with microsomes from *Candida albicans*. Moreland *et al.* (1996) reported that Avocado microsomes show typical Type I binding with Prosulfuron. The Cyt.P-450 from fungus *Ustilago maydis* showed Type II binding of tetraconazole, indicating coordination between triazole N-4 and heme iron (Carelli *et al.*, 1992).

Jeffery (1991) reported that Cyt.P-450 content is highest in liver followed by that in kidney, lungs, gut-and nasal epithelium. Popp and Cattley (1991) observed that the exposure of liver to xenobiotics may result in non-toxic

hepatic responses which may simply be an attempt to adjust to the xenobiotics e.g. Phenobarbital produces 2-5 fold increase in SER or Cyt.P-450 monooxygenase activity.

In general, calculated (Table 13) functional Cyt.P-450 content (difference in absorbance at 450 nm and 500 nm mg^{-1} microsomal protein) of hepatic microsomes was decreased by most of the pesticides except Phenthoate and Methyl parathion. The calculated functional Cyt.P-450 content of kidney microsomes was decreased by Phorate and Monocrotophos and increased by Ethion, Phenthoate, Malathion, Methyl parathion and Quinalphos, whereas Dimethoate had no such effect. This suggested that two pesticides and six pesticides may be more toxic to kidneys and liver respectively. All the pesticides except Phenthoate increased the calculated functional Cyt.P-450 content of microsomes from spleen. The results suggest better ability of spleen and kidneys to detoxify most of these pesticides. Phorate decreased the calculated functional Cyt.P-450 content of microsomes from brain, whereas other pesticides (except Monocrotophos and Ethion which had no effect) increased the proportion of calculated Cyt.P-450 content. The increase was considerably higher with Methyl parathion. It appears that microsomes from brain have varying ability to detoxify different pesticides. The calculated functional Cyt.P-450 content of testes was decreased by Phorate, Phenthoate, Malathion, Dimethoate and Monocrotophos but Methyl parathion increased their proportion. From the binding profile of the pesticides (Table 11) and the calculated Cyt.P-450 (Table 13) content of microsomes from testes, it appears that most of

Table 13. Change in AU($x10^{-3}$) at 450 nm and 500 nm mg^{-1} microsomal proteins from different tissues of goats by 250 μg a.i. of organophosphorus pesticides

	Liver	Kidney	Spleen	Brain	Testes
Control	3.20	3.74	1.58	4.27	4.44
Ethion	2.37 (-0.83)	7.48 (+3.74)	2.37 (+0.79)	4.27 (NC)	4.44 (NC)
Phorate	2.37 (-0.83)	1.87 (-1.87)	3.16 (+1.58)	2.85 (-1.42)	3.33 (-1.11)
Phenthoate	3.20 (NC)	6.55 (+2.81)	1.58 (NC)	5.70 (+1.43)	3.33 (-1.11)
Malathion	2.37 (-0.83)	6.55 (+2.81)	4.74 (+3.16)	4.99 (+0.72)	1.11 (-3.33)
Methyl parathion	3.56 (+0.30)	6.55 (+2.81)	4.74 (+3.16)	11.0 (+6.73)	5.55 (+1.11)
Quinalphos	2.77 (-0.43)	4.68 (+0.94)	3.16 (+1.58)	7.83 (+3.56)	4.44 (NC)
Dimethoate	2.37 (-0.83)	3.74 (NC)	2.37 (+0.79)	7.12 (+2.85)	2.22 (-2.22)
Monocrotophos	2.37 (-0.83)	2.80 (-0.94)	2.37 (+0.79)	4.27 (NC)	3.33 (-1.11)

Values in parentheses indicate increase(+) or decrease(-) over the control after binding to microsomes; values represent calculated functional Cyt.P-450; NC, no change

these pesticides adversely affect male reproductive functions. Ahuja *et al.*(1997) reported that Monocrotophos (19.6%) and Phorate (11%) produced extensive microsomal degranulation of rat liver cells under *in vitro* conditions. Whereas Quinalphos, Dimethoate and Methyl parathion produced minor (5 and 6%) microsomal degranulation. Such results also suggest changes around heme of hemoprotein of microsomes. From the *in vivo* and *in vitro* results, Ahuja *et al.*(1997) concluded that prolonged oral administration of non-lethal doses of Phorate and Monocrotophos have carcinogenic potential.

4.3.2 Absolute Absorption Spectra of Microsomes with Pesticides

Absolute Absorption Spectra of mixtures of pesticides with microsomes from different tissues of goats were recorded between 350 nm to 575 nm (Table 14, Fig.17,18).

4.3.2.1 Ethion (Fig.17): Ethion produced hyperchromic changes in the Absolute Absorption Spectra of microsomes from kidney, brain, spleen, testes and liver. Except for Type-I binding to microsomes from spleen, the binding of Ethion was of Type II and Reverse Type I with microsomes from liver, brain and testes (Table 11). These results suggested that Ethion produces direct effects mainly by binding to the heme of microsome components from brain, liver, kidney and testes and indirectly by binding to microsomes from spleen.

Unlike the other pesticides Phenthoate and Malathion produced hypochromic effect on the Absolute Absorption Spectra of microsomes from liver.

4.3.2.2 Phorate (Fig.17): The microsomes especially from kidneys, brain, testes and spleen showed much higher

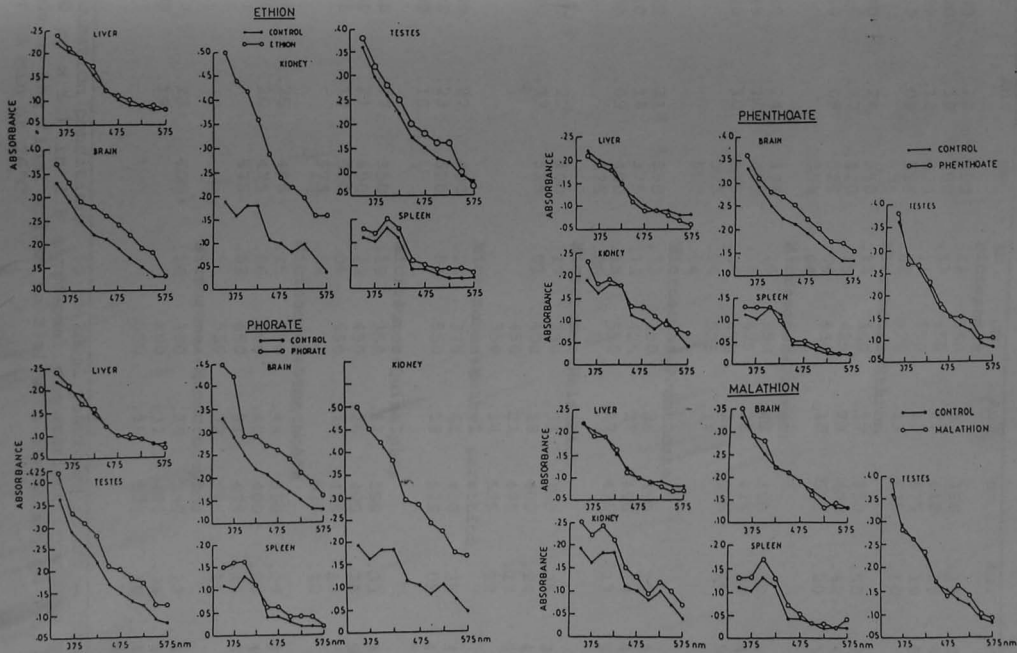


Fig.17. ABSOLUTE ABSORPTION SPECTRA OF DIFFERENT ORGANOPHOSPHORUS PESTICIDES (250 μg a. i.) WITH MICROSOMES FROM DIFFERENT TISSUES OF GOATS

Table 14. Effect of organophosphorus pesticides on the Absolute Absorption Spectra of microsomes from different tissues of goats

Liver		Spleen		Brain		Kidneys		Testes	
nm	Au	nm	Au	nm	Au	nm	Au	nm	Au
			Microsomes						
350	.22	350	.11	350	.33	350	.19	350	.36
400	.19	400	.13	450	.21	412	.18	425	.22
450	.12	425	.11	550	.13	475	.10	525	.12
550	.09	525	.02	575	.13	525	.10		
			Microsomes+Ethion						
350	.24	350	.13	350	.37	350	.50	350	.38
425	.17	400	.15	425	.28	400	.42	425	.25
550	.09	525	.04	550	.18	525	.20	525	.16
		550	.04						
			Microsomes+Phorate						
350	.24	375	.16	350	.42	350	.50	350	.42
425	.16	400	.16	425	.26	425	.38	425	.28
500	.10	475	.06	475	.20	525	.10	525	.17
		525	.04	525	.17				
		550	.04						
			Microsomes+Phenthoate						
350	.21	350	.13	350	.36	350	.23	350	.38
400	.18	375	.13	425	.27	400	.19	400	.27
500	.09	400	.13	550	.17	475	.13	500	.15
		475	.05						
			Microsomes+Malathion						
350	.22	400	.17	350	.35	350	.25	350	.39
400	.19	525	.03	400	.28	400	.24	400	.26
				450	.21	475	.13	500	.16
				550	.14	525	.12		
			Microsomes+Methyl parathion						
350	.27	400	.24	375	.61	400	.35	350	.54
400	.23	500	.04	550	.13	525	.09	400	.50
500	.10							500	.18
			Microsomes+Quinalphos						
350	.27	350	.19	350	.61	350	.33	350	.47
400	.21	400	.19	450	.26	400	.28	500	.15
500	.10	500	.04	550	.16	475	.13		
		575	.04			525	.10		
			Microsomes+Dimethoate						
350	.25	400	.15	350	.46	350	.27	350	.44
400	.20	475	.06	400	.40	400	.26	425	.39
		575	.03	450	.31	475	.16	500	.20
				550	.22	525	.11		
			Microsomes+Monocrotophos						
350	.23	400	.14	350	.38	350	.22	350	.37
400	.19			400	.31	400	.21	400	.27
				550	.16	525	.10	425	.24
								500	.16

Incubation conditions: 50 to 100μl pesticide solution (providing 250 μg a.i.) in 1.8 microsomal preparations from 1.8g tissue and made up the final volume to 2 ml with ST-buffer, pH 7.5, incubated for 15 min at 26°C

hyperchromic effect between 350 nm to 575 nm in the presence of Phorate, whereas, those from liver did not show such effect. These results suggested that Phorate disorganises the microsomes from kidney, brain, testes and spleen in this decreasing order. The differences in disorganisation of microsomes from different organs suggested differences in the components of microsomes from these organs which can interact with Phorate. Binding of Phorate to microsomes from kidney and spleen was of Type I indicating interaction with lipophillic and non-heme sites. Whereas the binding (Table 11) with microsomes of liver, brain and testes was of Type II and Reverse Type I. Such interactions suggested binding of Phorate to the heme iron in these tissues. Therefore, in the presence of Phorate, the cytochrome components of the microsomes from liver, brain and testes may be inactivated by binding to heme, whereas those from spleen and kidneys may or may not do so, depending on the indirect effects of microsomal changes.

4.3.2.3 Phenthoate (Fig.17): Phenthoate produced hyperchromic changes in the Absolute Absorption Spectra of microsomes from brain and kidneys. The hyperchromic effect with microsomes of spleen and testes was of lower magnitude. Phenthoate showed Type I binding with microsomes from spleen, kidneys and testes; Type II and Reverse Type I binding with microsomes from liver. It appears that in general, Phenthoate produced these effects by binding to non-heme components of microsomes except those of liver in which effects are due to the binding to heme components of microsomes.

4.3.2.4 Malathion (Fig.17): Malathion produced hyperchromic

changes in the Absolute Absorption Spectra of microsomes from kidney, spleen and testes (especially at 425 nm to 475 nm). Malathion produced hyperchromic and hypochromic changes in the Absolute Absorption Spectra in UV and visible range, respectively, of microsomes from brain. Binding of Malathion with microsomes of spleen and brain is of Type I; Type II and Reverse Type I with microsomes of liver and testes; Reverse Type I with microsomes of kidneys (Table 11). The results suggest that Malathion indirectly influences the microsomes from spleen and brain by binding to lipophillic site whereas in liver, testes and kidneys the effects are likely to be produced by direct binding to heme component of the microsomes.

4.3.2.5 Methyl parathion (Fig.18): Like Phorate, in the presence of Methyl parathion microsomes from brain, kidneys, testes, spleen and liver showed hyperchromic changes in decreasing order, which suggested that Methyl parathion disorganises the microsomes. Binding of Methyl parathion to microsomes from brain and testes was of Type I; to those from liver and spleen was of Reverse Type I and to those of kidney the binding was of Reverse Type I and Type II (Table 11). Such results suggested that binding of Methyl parathion to heme component of microsomes from kidneys was higher compared to those from liver and spleen.

4.3.2.6 Quinalphos (Fig.18): Quinalphos produced hyperchromic changes in the Absolute Absorption Spectra of microsomes from brain, kidney, spleen, testes and liver in decreasing order. This suggested that Quinalphos results in the disorganisation of microsomes from these organs. Binding of Quinalphos to

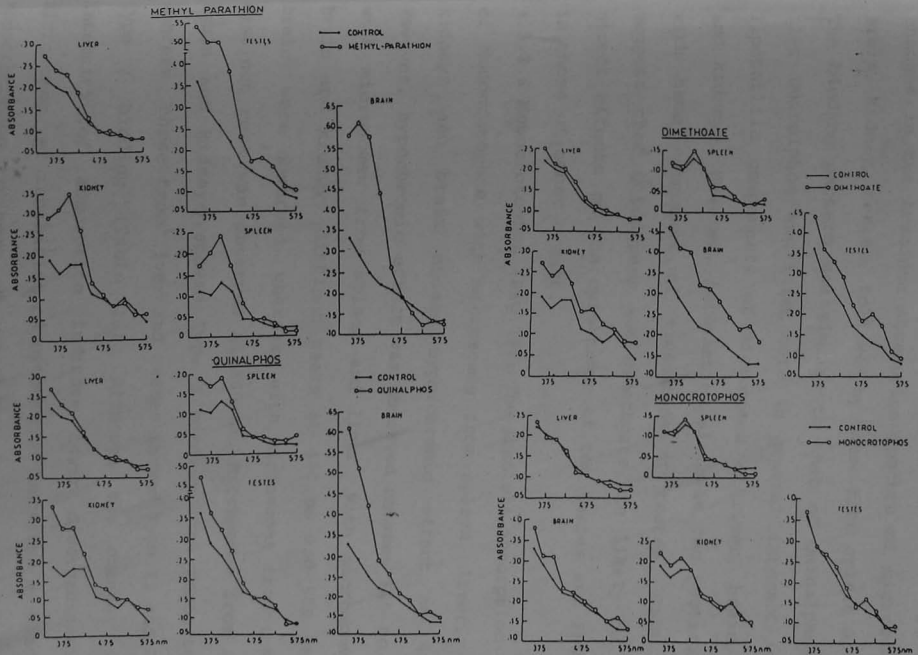


Fig. 18 ABSOLUTE ABSORPTION SPECTRA OF DIFFERENT ORGANOPHOSPHORUS PESTICIDES (250 µg a. i.) WITH MICROSOMES FROM DIFFERENT TISSUES OF GOATS

microsomes from liver, spleen, brain and kidneys was of Type I while with those from testes was of Type II and Reverse Type I (Table 11).

4.3.2.7 Dimethoate (Fig.18): Dimethoate produced hyperchromic changes in the Absolute Absorption Spectra of microsomes from brain, kidney, testes, followed by those from spleen and liver. The binding pattern was similar to that of Quinalphos (Table 11). Quinalphos and Dimethoate in general interact with the lipophillic components of microsomes of liver, brain, spleen and kidneys. However, interaction of these two pesticides was with heme component of microsomes of testes. Such results suggest that Quinalphos and Dimethoate are likely to produce direct effects on the cytochrome of the testes and indirectly in those of other organs.

4.3.2.8 Monocrotophos (Fig.18): The Absolute Absorption Spectra of Monocrotophos with microsomes from spleen, liver, testes, kidneys and brain showed hyperchromic effect at 400 nm. However, hypochromic effect was observed between 525 to 575 nm with microsomes from spleen and liver. With microsomes from brain and kidney, distinct peaks at 400 nm and 550 nm (only brain) were observed, whereas, with microsomes from testes, a distinct peak was observed at 500 nm. Microsomes from spleen, brain and kidneys showed Type I binding with Monocrotophos, whereas, those from liver and testes showed Type II and Reverse Type I binding (Table 11). Although the changes in the intensity of absorption indicate lower disorganisation of microsomes yet inhibition of cytochrome functions by binding of Monocrotophos to heme of cytochrome in liver and testes is

evident. Whereas the effects in microsomes of spleen, brain and kidneys appeared to be indirect, involving the non-heme components of microsomes.

4.3.3 Kinetics of Microsomal Esterases

Yan *et al.*(1995) designated esterases from liver microsomes of rats as A, B, C, S and egasyn. Aldridge (1953) reported that esterases A hydrolyse the organophosphorus pesticides and esterases B are inhibited by pesticides. Effect of pH, temperature and substrate concentration on the activity of total esterases from microsomes of tissues of goats was studied in absence and presence of pesticides. Therefore, it is not possible to distinguish the relative activities of two types of esterases. However, the universal presence of these two esterases in the preparations of microsomes from different tissues could be deduced.

4.3.3.1 Effect of pH (Figs.19-21): The duodenal- and abomasal mucosal esterases and microsomal esterases from brain showed maximum activities at pH 8.0-8.5, pH 8.0-8.5 and pH 8.0, respectively. The microsomal esterases from testes, liver, kidneys and spleen showed maximum activity in decreasing order at pH 7.4. At pH 7.4, the activity of these esterases was still high in brain. These results suggested that degradation of ester linkages of organophosphorus pesticides in brain and small intestine will be high. This also shows that the microsomal esterases from different tissues show higher activities with increasing pH.

4.3.3.2 Effect of Temperature (Figs.19-21): The abomasal-and duodenal mucosal esterases and microsomal esterases from liver,

kidney, testes, brain showed maximal activity at 37°C, whereas microsomal esterases from spleen showed maximal activity at 45°C. At these temperatures the higher activity of microsomal esterases from brain, kidneys and liver was observed; and those from testes showed slightly lower activities. The microsomal esterases from all these tissues were active at 25 to 55°C. Comparison of Arrhenius plots of microsomal esterases from liver, spleen, kidneys and brain suggested that esterases were inactivated at temperatures between 37°C- 45°C whereas those from abomasal mucosal cells, testes and duodenal mucosal cells were relatively stable. The activation energy (Ea) of microsomal esterases from spleen, kidneys, testes, liver and brain were 6072, 6831, 7970, 11021 and 14801 cal $mole^{-1}$, respectively, whereas the esterases from abomasal- and duodenal mucosal cells showed Ea values of 3038 and 4554 cal $mole^{-1}$, respectively.

The results suggest that inspite of higher V_{max} of microsomal esterases from brain, the modification of pesticides in the brain may be low when energy is a limiting factor, so during toxicity brain functions may be adversely affected. The higher Ea of microsomal esterases from liver indicates higher needs of energy which may be met efficiently not only from glucose but also from other metabolites available in liver. Therefore, the modification of pesticides in liver may be continued for longer time compared to that in brain. In duodenal mucosal cells, kidneys and splenocytes, the Ea values for esterases are low and these esterases are active at broad range of temperature, suggesting that modification of

pesticides in these tissues may be faster and without complete inactivation of esterases.

4.3.3.3 Effect of Substrate Concentrations (Figs.19-21): The effect of substrate concentrations on the microsomal esterases from tissues of goats is represented as Michaelis-Menten plot and its linear transformations viz. Lineweaver-Burk, Hanes and Eadie-Hofstee plots. The results suggested that the presence of at least two esteraes in abomasal-, duodenal mucosal cells and in microsomes from liver, kidneys, testes, brain and spleen.

The shape of Lineweaver-Burk plots suggested that duodenal- and abomasal mucosal esterases and the microsomal esterases from liver, kidney, brain, spleen and testes are inhibited by substrate concentration > 0.8 μM. Wilson and Walker (1997) concluded that the Lineweaver-Burk equation tends to give an uneven distribution of points of substrate concentration and suggested that Hanes and Eadie-Hofstee plots should be used instead. As better linearity was obtained with Hanes plots, so the Km and Vmax values from such plots for microsomal esterases from various organs were compared (Table 15). It appears that esterases from duodenal mucosal cells, kidney, testes and liver microsomes have both very low and high Km values, suggesting that these esterases respectively have high and low affinities for substrate and represent different protein as enzymes. The esterases from duodenal mucosal cells are apparently different from those in the kidneys and testes. The esterases in liver are different, more with regard to their Vmax values and less with regard to Km values i.e. affinities

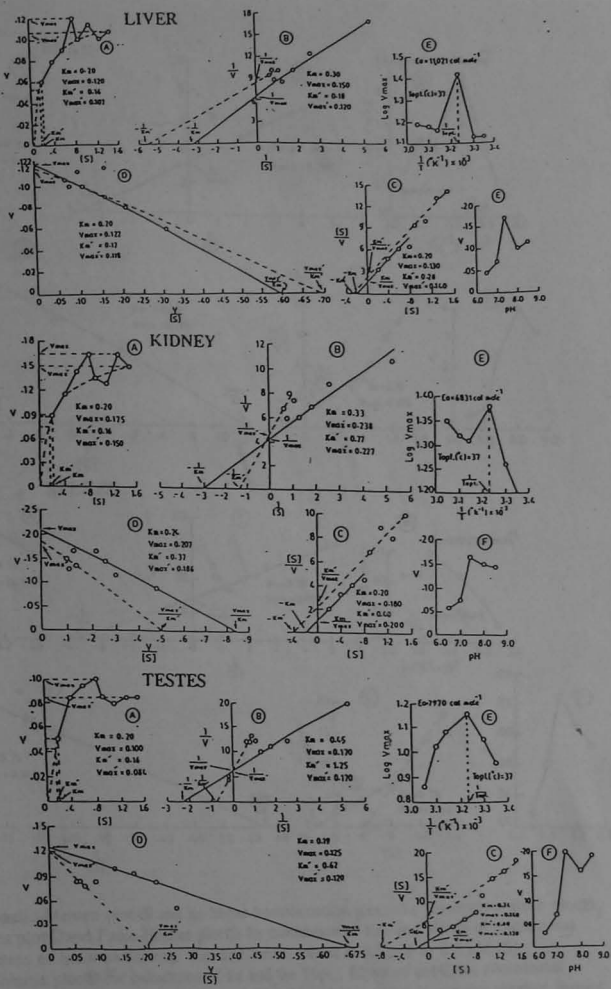


Fig.19. Michaelis-Menten plot Ⓐ and its linear transformation plots viz. Lineweaver-Burk plot Ⓑ, Hanes plot Ⓒ and Eadie-Hofstee plot Ⓓ for calculation of Km and Vmax of microsomal esterases of liver, kidney and testes. Km and Vmax, values at 0.2 μM to 0.8 μM [S]; Km′ and Vmax′ values at 0.9 μM to 1.5 μM [S]; Arrehinius plot Ⓔ for calculation of Ea and the Topt.; Effect of pH Ⓕ on microsomal esterases of liver, kidney and testes. Km and [S], μM and Vmax and V, μmoles product formed h^{-1} mg^{-1} microsomal proteins.

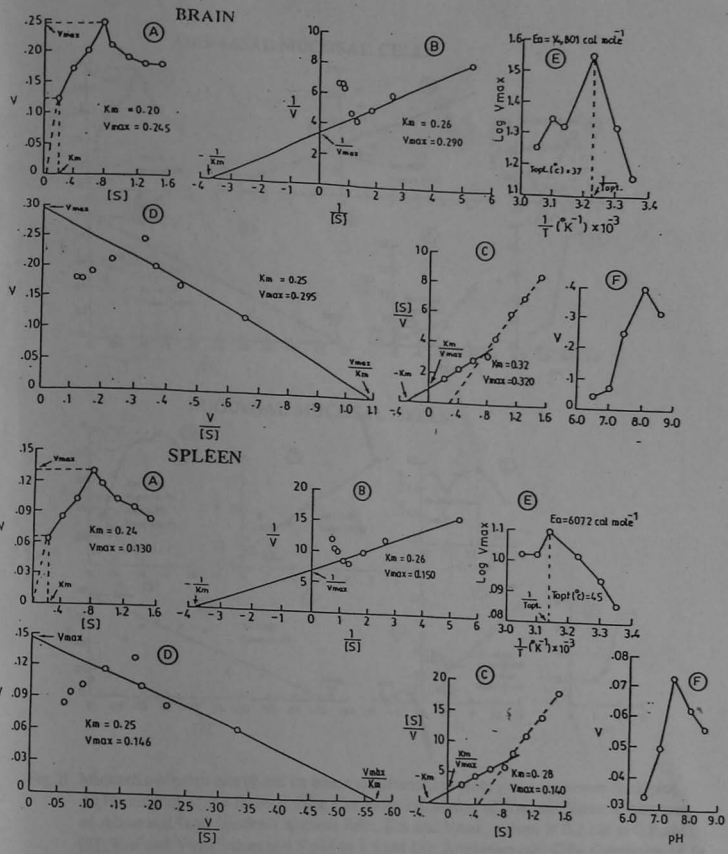


Fig. 20. Michaelis-Menten plot Ⓐ and its linear transformation plots viz Lineweaver-Burk plot Ⓑ, Hanes plot Ⓒ and Eadie-Hofstee plot Ⓓ for calculation of Km and Vmax of microsomal esterases of brain and spleen. Km and Vmax, values at 0.2 μM to 0.8 μM [S]; Arrehinius plot Ⓔ for calculation of Ea and the Topt.; Effect of pH Ⓕ on microsomal esterases of brain and spleen. Km and [S], μM and Vmax and V, μmoles product formed $h^{-1} mg^{-1}$ microsomal proteins.

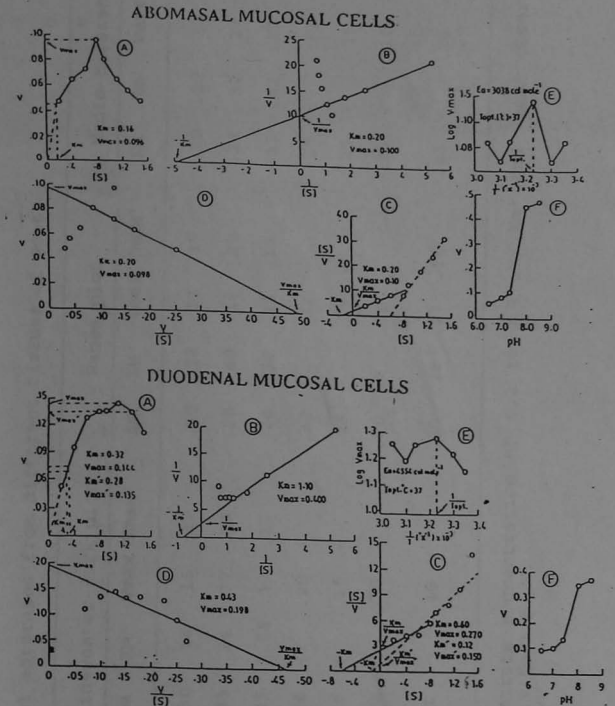


Fig.21. Michaelis-Menten plot Ⓐ and its linear transformation plots viz. Lineweaver-Burk plot Ⓑ, Hanes plot Ⓒ and Eadie-Hofstee plot Ⓓ for calculation of Km and Vmax of esterases of Abomasal- and Duodenal mucosal cells. Km and Vmax, values at 0.2 μM to 0.8 μM [S]; Km and Vmax values at 0.9 μM to 1.5 μM [S]; Arrehinius plot Ⓔ for Calculation of Ea and the Topt.; Effect of pH Ⓕ on esterases of abomasal-duodenal mucosal cells. Km and [S], μM and Vmax and V, μmoles product formed h^{-1} mg^{-1} proteins.

Table 15. Km and Vmax of microsomal esterases from different tissues of goats

Tissues	Michaelis-Menten plot				Lineweaver-Burk plot				Hanes plot				Eadie-Hofstee plot			
	Km	Km'	Vmax	Vmax'	Km	Km'	Vmax	Vmax'	Km	Km'	Vmax	Vmax'	Km	Km'	Vmax	Vmax'
Liver	.20	.16	.12	.11	.30	.18	.15	.12	.20	.28	.13	.19	.20	.17	.12	.12
Kidney	.20	.16	.18	.15	.33	.24	.77	.23	.20	.40	.18	.20	.24	.37	.21	.19
Testes	.20	.16	.10	.08	.45	1.25	.17	.17	.24	.80	.16	.13	.19	.62	.13	.12
Brain	.20	-	.25	-	.26	-	.29	-	.32	-	.32	-	.25	-	.30	-
Spleen	.24	-	.13	-	.26	-	.15	-	.28	-	.14	-	.25	-	.15	-
Abomasal mucosa	.16	-	.10	-	.20	-	.10	-	.20	-	.10	-	.20	-	.10	-
Duodenal mucosa	.32	.28	.14	.14	1.1	-	.40	-	.60	.12	.27	.15	.43	-	.20	-

Km and Vmax observed at lower substrate concentration of 0.2 to 0.8 μM; Km' and Vmax' observed at higher subtrate concentrations of 0.9 to 1.5 μM.

for the substrate. The esterases from abomasal mucosal cells have both low Km and low Vmax values. It was concluded that esterases are the sites for sequestering of the pesticides. Suzuki *et al.*(1993) reported that carboxylesterase acts as a sequestering protein during Fenitrothion resistance.

Within the cells these esterases may be responsible for initial modification of ingested and absorbed pesticides. The esterases from abomasal mucosal cells has higher affinity for the substrate. The microsomal esterases from brain showed higher Km and Vmax values indicating lower affinity with higher activity and thus the latter compensates for the former. Thus, it may be more effective in modifying the effects of pesticides on brain.

Wilson and Walker (1997) concluded that Lineweaver-Burk plot with an upward curve at high substrate concentration (low 1/[S]values)indicates substrate inhibition. The Lineweaver Burk plots of abomasal- and duodenal mucosal esterases and of microsomal esterases (Figs.19-21) from liver, kidney, brain, spleen and testes showed such curves indicating substrate inhibition at substrate concentration >0.8 μM.

4.3.3.4 Inhibition of Microsomal Esterases

4.3.3.4.1 Hepatic Microsomal Esterases: Inhibition of hepatic microsomal esterases by Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos was relatively higher compared to those of kidney, spleen, brain, testes, abomasal and duodenal mucosal cells (Fig.22,23, Tables 16,17). In general, with increasing concentration of pesticides, there was higher inhibition of esterases at all

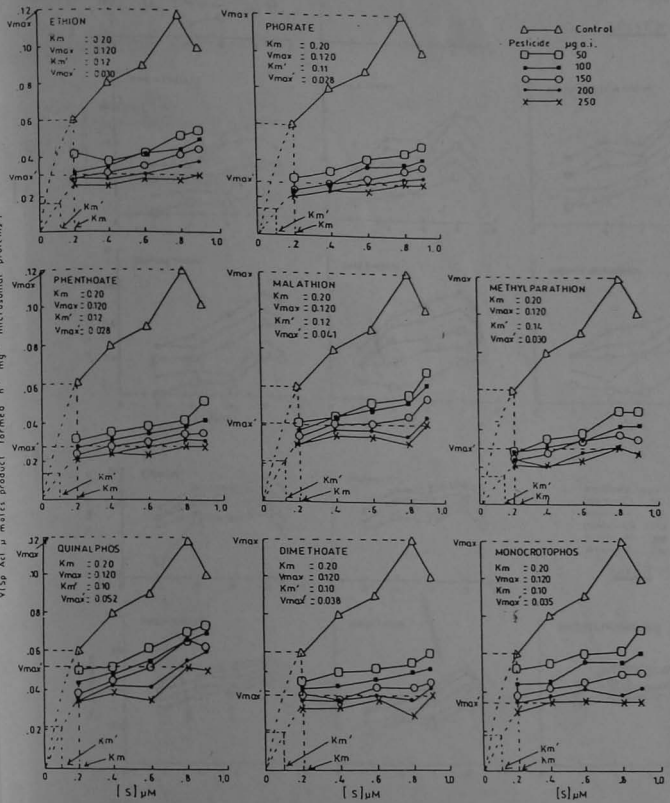


Fig 22 Effect of concentration of different organophosphorus pesticides (µg a.i.) on the Michaelis–Menten curve of microsomal esterase (s) from Liver of goat (Km and Vmax, control values; and Km' and Vmax', values in the presence of 250 µg a.i.)

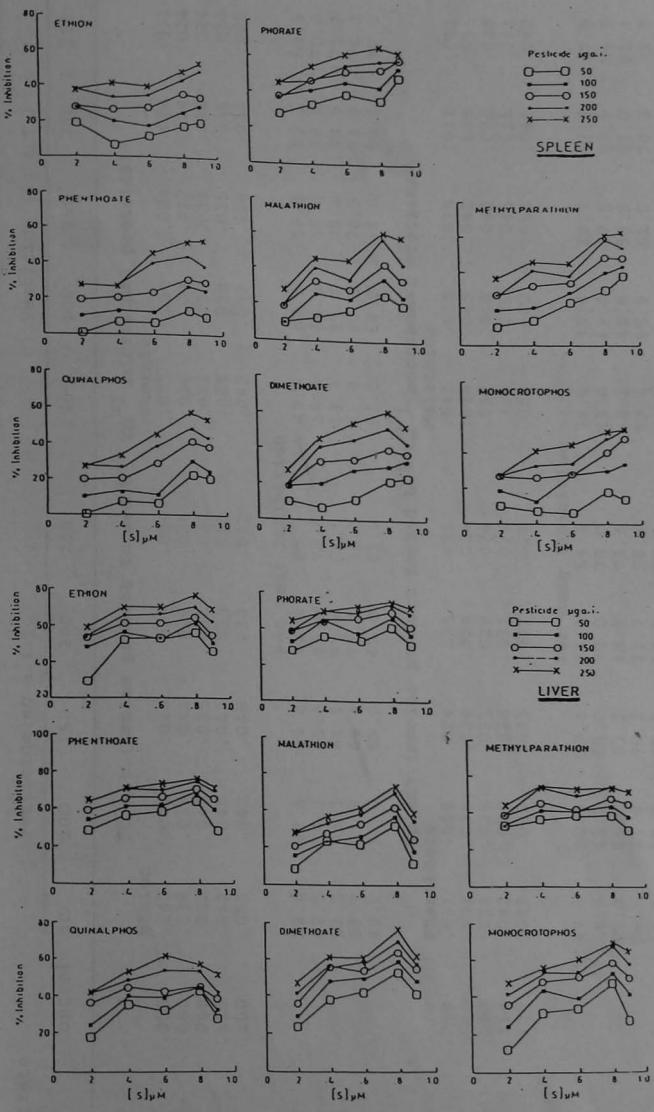


Fig. 2 Effect of concentration of different organophosphorus pesticides (µg a.i.) on Percent Inhibition of microsomal esterase(s) from Spleen and Liver

Table 16. *In vitro* inhibition of esterases from microsomes of liver of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate		Pesticide (μg a.i.)					Pesticide (μg a.i.)				
(μM)	Control	50	100	150	200	250	50	100	150	200	250
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Ethion					Phorate				
.2	.059	.042	.031	.028	.028	.024	.031	.028	.024	.024	.021
.4	.080	.038	.035	.031	.028	.024	.035	.028	.028	.024	.024
.6	.090	.042	.042	.035	.031	.028	.042	.038	.031	.028	.024
.8	.118	.052	.045	.042	.035	.028	.045	.038	.035	.031	.028
.9	.100	.055	.049	.045	.038	.031	.049	.042	.038	.031	.028
		Per cent Inhibition									
.2		28.8	47.5	52.5	52.5	59.3	47.5	52.5	59.3	59.3	64.4
.4		52.5	56.3	61.3	65.0	70.0	56.3	65.0	65.0	70.0	70.0
.6		53.3	53.3	61.1	65.6	68.9	53.3	57.8	65.6	68.9	73.3
.8		55.9	61.9	64.4	70.3	76.3	61.9	67.8	70.3	73.7	76.3
.9		45.0	51.0	55.0	62.0	69.0	51.0	58.0	62.0	69.0	72.0
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Phenthoate					Malathion				
.2	.059	.031	.028	.024	.021	.021	.042	.038	.035	.031	.031
.4	.080	.035	.031	.028	.024	.024	.045	.045	.042	.038	.035
.6	.090	.038	.035	.031	.028	.024	.052	.049	.042	.038	.035
.8	.118	.042	.038	.035	.031	.028	.055	.052	.045	.035	.031
.9	.100	.052	.042	.035	.031	.028	.069	.062	.055	.045	.042
		Per cent Inhibition									
.2		47.5	52.5	59.3	64.4	64.4	28.8	35.6	40.7	47.5	47.5
.4		56.2	61.2	65.0	70.0	70.0	43.8	43.8	47.5	52.5	56.5
.6		57.8	61.1	65.5	68.9	73.3	42.2	45.6	53.3	57.8	61.1
.8		64.4	67.8	70.3	73.7	76.3	53.4	55.9	61.9	70.3	73.7
.9		48.0	58.0	65.0	69.0	72.0	31.0	38.0	45.0	55.0	58.0

Table 17. *In vitro* inhibition of esterases from microsomes of liver of goats by 50 μg to 250 μg a.i. of organophosphorus pesticide

Substrate (μM)	Control	Pesticide (μg a.i.) 50	100	150	200	250	Pesticide (μg a.i.) 50	100	150	200	250
		Sp.Act (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Methyl parathion					Quinalphos				
.2	.059	.028	.028	.024	.024	.021	.049	.045	.038	.035	.035
.4	.080	.035	.031	.028	.021	.021	.052	.049	.045	.042	.038
.6	.090	.038	.035	.035	.028	.024	.062	.055	.052	.042	.035
.8	.118	.049	.042	.038	.031	.031	.069	.066	.066	.055	.052
.9	.100	.049	.042	.035	.028	.028	.073	.069	.062	.059	.049
		Per cent Inhibition									
.2		52.5	52.5	59.3	59.3	64.4	16.9	23.7	35.6	40.7	40.7
.4		56.3	61.3	65.0	73.8	73.8	35.0	38.8	43.8	47.5	52.5
.6		57.8	61.1	61.1	68.9	73.3	31.1	38.9	42.2	53.3	61.1
.8		58.5	64.4	67.8	73.7	73.7	41.5	44.1	44.1	53.4	55.9
.9		51.0	58.0	65.0	72.0	72.0	27.0	31.0	38.0	41.0	51.0
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Dimethoate					Monocrotophos				
.2	.059	.045	.042	.038	.035	.031	.052	.045	.038	.035	.031
.4	.080	.049	.042	.035	.035	.031	.055	.045	.042	.038	.035
.6	.090	.052	.045	.042	.038	.035	.059	.055	.045	.042	.035
.8	.118	.055	.049	.042	.035	.028	.062	.055	.049	.038	.035
.9	.100	.059	.052	.045	.042	.038	.073	.059	.049	.042	.035
		Per cent Inhibition									
.2		23.7	28.8	35.6	40.7	47.5	11.9	23.7	35.6	40.7	47.5
.4		38.8	47.5	56.3	56.3	61.3	31.3	43.8	47.5	52.5	56.3
.6		42.2	50.0	53.3	57.8	61.1	34.4	38.9	50.0	53.3	61.1
.8		53.4	58.5	64.4	70.3	76.3	47.5	53.4	58.5	67.8	70.3
.9		41.0	48.0	55.0	58.0	62.0	27.0	41.0	51.0	58.0	65.0

concentrations of the substrate (with few exceptions). At substrate concentration > 0.8 μM the esterase activity was inhibited even in the absence of pesticides. Such results indicate substrate inhibition of hepatic microsomal esterases. This was also indicated by the upward curve of Lineweaver Burk plot (Fig.19) at higher substrate concentration. However, this substrate inhibition was not observed with lower concentrations of pesticides. All concentrations of Malathion, Dimethoate, Ethion, Phorate showed increased activity at substrate concentration > 0.8 μM. With other pesticides similar results or lack of such effects was observed under similar conditions. It was concluded that all these pesticides inhibit the microsomal esterases of liver. These studies suggested that liver possesses esterases which hydrolyse and/or sequester these pesticides. The proportion of esterases which are inhibited by these pesticides appear to be higher. Malathion which possesses carboxylester linkages appears to be hydrolysed to greater degree by hepatic microsomal esterases. Methyl parathion, Phorate and Phenthoate produced maximal inhibition of hepatic microsomal esterases. Chambers and Chambers (1990) reported that the plasma carboxylesterases were maximally inhibited within 15 min after administration of organophosphorus insecticides.

4.3.3.4.2 Brain Microsomal Esterases: The microsomal esterases from brain showed inhibition with substrate concentration >0.8 μM in absence of pesticides. In the presence of various concentrations of pesticides, the microsomal esterases prepared from brain were inhibited maximally by Phorate, Methyl

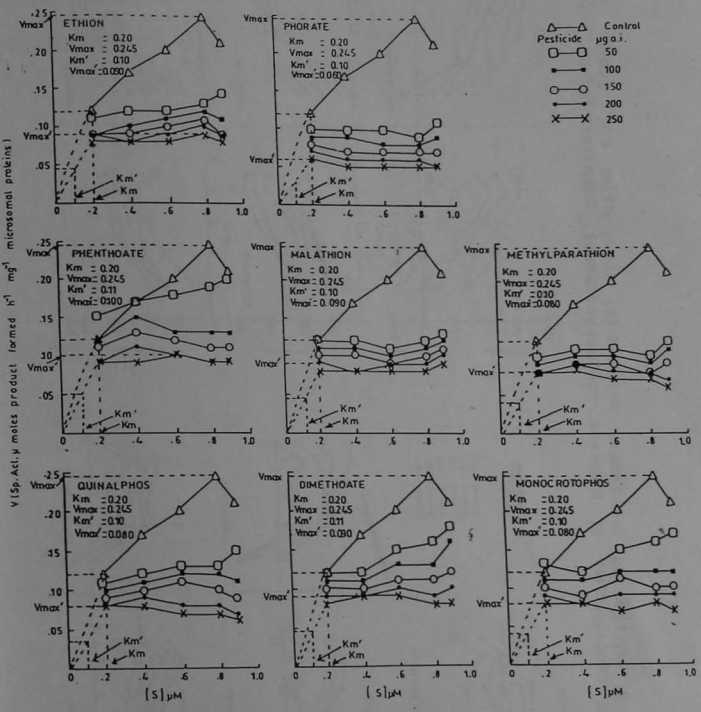


Fig. 24. Effect of concentration of different organophosphorus pesticides (µg a.i.) on the Michaelis–Menten curve of microsomal esterase (s) from Brain of goat (Km and Vmax, control values; and Km' and Vmax', values in the presence of 250 µg a.i.)

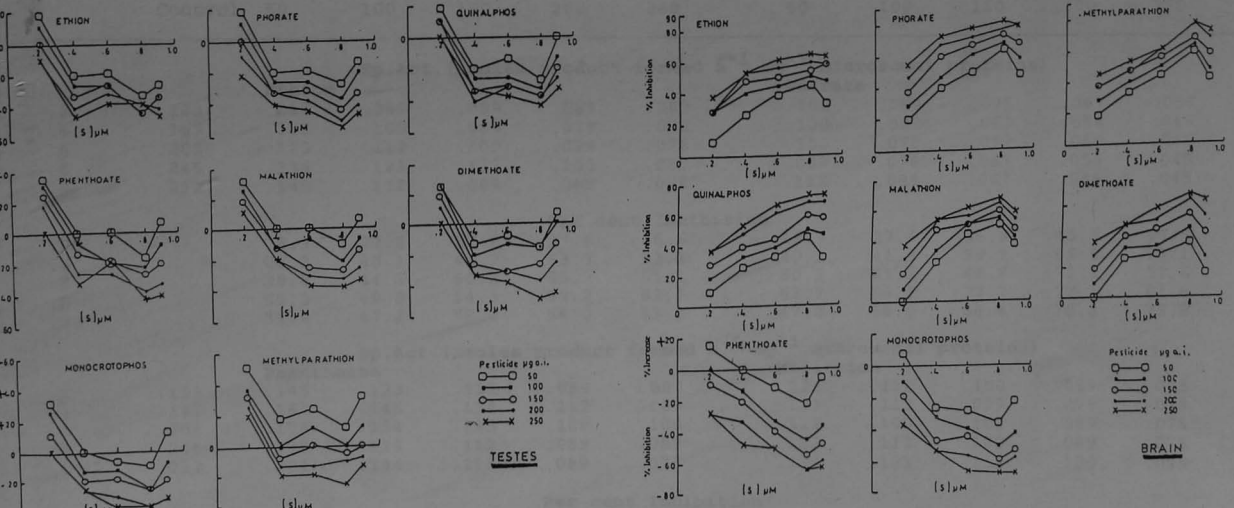


Fig. 25. Effect of concentration of different organophosphorus pesticides (µg a.i.) on Percent change in activity of microsomal esterase (s) from Testes and Brain.

Table 18. *In vitro* inhibition of esterases from microsomes of brain of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.) 50	100	150	200	250	Pesticide (μg a.i.) 50	100	150	200	250
		Sp.Act.(μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Ethion					**Phorate**				
.2	.123	.112	.089	.089	.089	.089	.100	.089	.078	.067	.056
.4	.167	.123	.100	.089	.078	.078	.100	.089	.067	.056	.045
.6	.201	.123	.112	.100	.089	.078	.100	.078	.067	.056	.045
.8	.245	.134	.123	.112	.100	.089	.089	.078	.067	.056	.045
.9	.212	.145	.112	.089	.089	.078	.112	.089	.067	.045	.045
		Per cent Inhibition									
.2		8.9	27.6	27.6	27.6	36.6	18.7	27.6	36.6	45.5	54.5
.4		26.3	40.1	46.7	53.3	53.3	40.1	46.7	59.9	66.5	73.1
.6		38.8	44.3	50.2	55.7	61.2	50.2	61.2	66.7	72.1	77.6
.8		45.3	49.8	54.3	59.2	63.7	63.7	68.2	72.7	77.1	81.6
.9		31.6	47.2	58.0	58.0	63.2	47.2	58.0	68.4	78.8	78.8
		Sp.Act.(μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Phenthoate					**Malathion**				
.2	.123	.145	.123	.112	.089	.089	.123	.112	.100	.089	.078
.4	.167	.167	.145	.134	.112	.089	.123	.112	.089	.078	.078
.6	.201	.178	.134	.123	.100	.100	.112	.100	.100	.089	.078
.8	.245	.190	.134	.112	.089	.089	.123	.112	.100	.089	.078
.9	.212	.201	.134	.112	.089	.078	.134	.123	.112	.100	.089
		Per cent Inhibition									
.2		+15.2	-	8.9	27.6	27.6	-	8.9	18.7	27.6	36.6
.4		-	13.2	19.8	32.9	46.7	26.3	32.9	46.7	53.3	53.3
.6		11.4	33.3	38.8	50.2	50.2	44.3	50.2	50.2	55.7	61.2
.8		22.4	45.3	54.3	63.7	63.7	49.8	54.3	59.2	63.7	68.2
.9		5.2	36.8	47.2	58.0	63.2	36.8	42.0	47.2	52.8	58.0

+, indicates an unexplained increase in activity in presence of the pesticide

Table 19. *In vitro* inhibition of esterases from microsomes of brain of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.) 50	100	150	200	250	Pesticide (μg a.i.) 50	100	150	200	250
		Sp.Act.(μmoles product formed h^{-1} mg^{-1} microsomal protein)									
		Methyl parathion					Quinalphos				
.2	.123	.100	.089	.078	.078	.067	.112	.100	.089	.078	.078
.4	.167	.112	.100	.089	.089	.078	.123	.112	.100	.089	.078
.6	.201	.112	.100	.089	.078	.078	.134	.123	.112	.078	.067
.8	.245	.100	.089	.078	.056	.056	.134	.123	.100	.078	.067
.9	.212	.123	.112	.089	.067	.056	.145	.112	.089	.067	.056
		Per cent Inhibition									
.2		18.7	27.6	36.6	36.6	45.4	8.9	18.7	27.6	36.6	36.6
.4		32.9	40.1	46.7	46.7	53.3	26.3	32.9	40.1	46.7	53.3
.6		44.3	50.2	55.7	61.2	61.2	33.3	38.8	44.3	61.2	66.7
.8		59.2	63.7	68.2	77.1	77.1	45.3	49.8	59.2	68.2	72.7
.9		42.0	47.2	58.0	68.4	73.6	31.6	47.2	58.0	68.4	73.6
		Sp.Act.(μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Dimethoate					Monocrotophos				
.2	.123	.123	.112	.100	.089	.078	.134	.112	.100	.089	.078
.4	.167	.123	.112	.100	.089	.089	.123	.112	.089	.078	.078
.6	.201	.145	.134	.112	.100	.089	.145	.123	.112	.089	.067
.8	.245	.156	.134	.112	.089	.078	.156	.123	.100	.089	.078
.9	.212	.178	.156	.123	.100	.078	.167	.123	.100	.089	.067
		Inhibition(%)									
.2		-	8.9	18.7	27.6	36.6	+8.2	8.9	18.7	27.6	36.6
.4		26.3	32.9	40.1	46.7	46.7	26.3	32.9	46.7	53.3	53.3
.6		27.9	33.3	44.3	50.2	55.7	27.9	38.8	44.3	55.7	66.7
.8		36.3	45.3	54.3	63.7	68.2	36.3	49.8	59.2	63.7	68.2
.9		16.0	26.4	42.0	52.8	63.2	21.2	42.0	52.8	58.0	68.4

+, indicates an unexplained increase in activity in presence of the pesticide

Table 19. *In vitro* inhibition of esterases from microsomes of brain of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.)					Pesticide (μg a.i.)				
		50	100	150	200	250	50	100	150	200	250
		Sp.Act.(μmoles product formed h^{-1} mg^{-1} microsomal protein)									
		Methyl parathion					Quinalphos				
.2	.123	.100	.089	.078	.078	.067	.112	.100	.089	.078	.078
.4	.167	.112	.100	.089	.089	.078	.123	.112	.100	.089	.078
.6	.201	.112	.100	.089	.078	.078	.134	.123	.112	.078	.067
.8	.245	.100	.089	.078	.056	.056	.134	.123	.100	.078	.067
.9	.212	.123	.112	.089	.067	.056	.145	.112	.089	.067	.056
		Per cent Inhibition									
.2		18.7	27.6	36.6	36.6	45.4	8.9	18.7	27.6	36.6	36.6
.4		32.9	40.1	46.7	46.7	53.3	26.3	32.9	40.1	46.7	53.3
.6		44.3	50.2	55.7	61.2	61.2	33.3	38.8	44.3	61.2	66.7
.8		59.2	63.7	68.2	77.1	77.1	45.3	49.8	59.2	68.2	72.7
.9		42.0	47.2	58.0	68.4	73.6	31.6	47.2	58.0	68.4	73.6
		Sp.Act.(μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Dimethoate					Monocrotophos				
.2	.123	.123	.112	.100	.089	.078	.134	.112	.100	.089	.078
.4	.167	.123	.112	.100	.089	.089	.123	.112	.089	.078	.078
.6	.201	.145	.134	.112	.100	.089	.145	.123	.112	.089	.067
.8	.245	.156	.134	.112	.089	.078	.156	.123	.100	.089	.078
.9	.212	.178	.156	.123	.100	.078	.167	.123	.100	.089	.067
		Inhibition(%)									
.2		-	8.9	18.7	27.6	36.6	+8.2	8.9	18.7	27.6	36.6
.4		26.3	32.9	40.1	46.7	46.7	26.3	32.9	46.7	53.3	53.3
.6		27.9	33.3	44.3	50.2	55.7	27.9	38.8	44.3	55.7	66.7
.8		36.3	45.3	54.3	63.7	68.2	36.3	49.8	59.2	63.7	68.2
.9		16.0	26.4	42.0	52.8	63.2	21.2	42.0	52.8	58.0	68.4

+, indicates an unexplained increase in activity in presence of the pesticide

parathion and Quinalphos (Fig.24, 25, Tables 18, 19). Inhibition by Malathion was increased if the substrate concentration was > 0.8 μM. Almost similar observations were made with Phorate, Methyl parathion and Monocrotophos. The inhibition of microsomal esterases from brain by Malathion does not correspond with LD_{50} dose of Malathion possibly due to the fact that Malathion is either degraded or sequestered in the digestive tract and liver. Ehrich *et al.*(1993) reported that the activities of brain and blood cholinesterase and carboxylesterase were more sensitive to inhibition by diisopropyl phosphorofluoridate than the hepatic enzymes. Chambers and Chambers (1990) reported that inhibition of rat brain esterase by Parathion and Paraoxon is higher than that of plasma.

4.3.3.4.3 Kidney Microsomal Esterases: The microsomal esterases from kidney showed inhibition by substrate concentration > 0.8 μM in the absence of pesticides. With various concentrations of Monocrotophos and Phenthoate (except 50 μg a.i.), substrate concentration >0.4 μM further decreased the activity of microsomal esterases from kidney (Tables 20,21, Figs.26,27). Similar results were obtained with Phorate (150 μg to 250 μg a.i.) at substrate concentration > 0.6 μM. Phorate, Quinalphos, Methyl parathion produced maximal inhibition. Kyle and Farber (1991) reported that extrahepatic tissues like kidneys, mucosa of gastrointestinal and respiratory tracts play important role in modification of ingested and absorbed compounds/xenobiotics.

4.3.3.4.4 Testicular Cell Microsomal Esterases: The microsomal esterases from testes showed inhibition by substrate

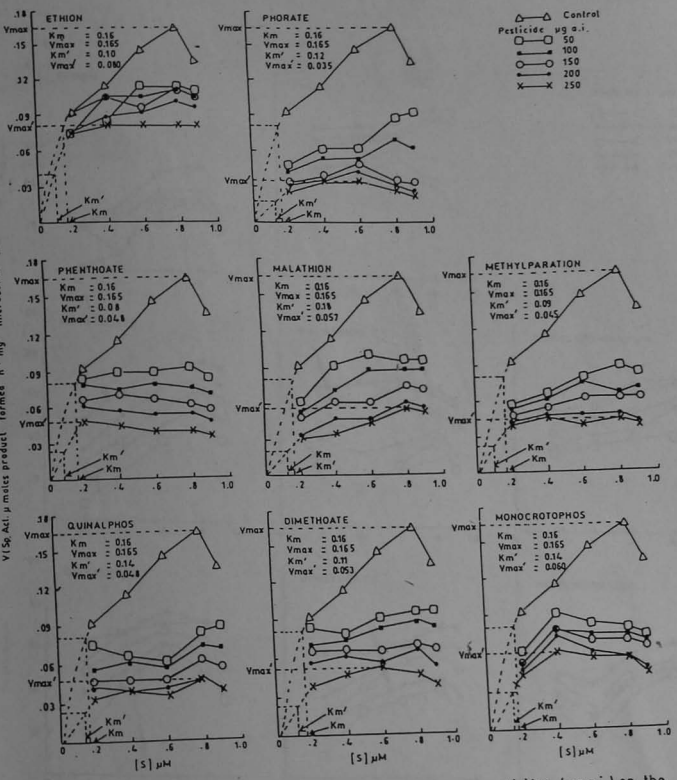


Fig. 26. Effect of concentration of different organophosphorus pesticides (µg a.i.) on the Michaelis–Menten curve of microsomal esterase (s) from kidneys of goat (Km and Vmax, control values; and Km' and Vmax', values in the presence of 250 µg a.i.)

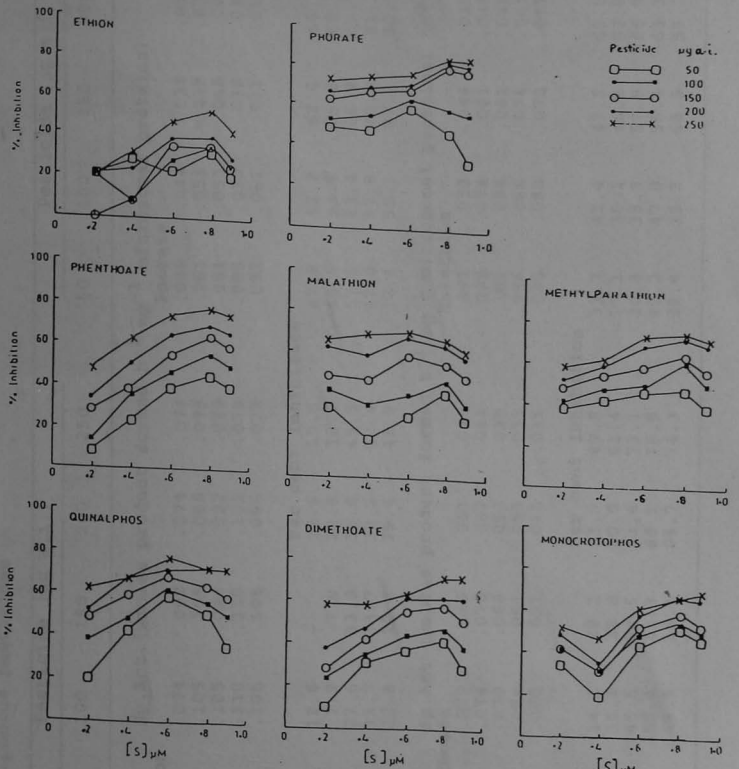


Fig.27.Effect of concentration of different organophosphorus pesticides (µg a.i.) on Percent Inhibition of microsomal esterase (s) from Kidney

Table 20. *In vitro* inhibition of esterases from microsomes of kidney of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate		Pesticide (μg a.i.)					Pesticide (μg a.i.)				
(μM)	Control	50	100	150	200	250	50	100	150	200	250
		Sp.Act.(μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Ethion					Phorate				
.2	.092	.074	.074	.092	.074	.074	.048	.044	.035	.031	.026
.4	.114	.083	.105	.105	.088	.079	.061	.053	.039	.035	.031
.6	.145	.114	.105	.096	.092	.079	.061	.053	.048	.044	.035
.8	.166	.114	.110	.110	.101	.079	.088	.070	.035	.031	.026
.9	.136	.110	.105	.105	.096	.079	.092	.061	.031	.026	.022
		Per cent Inhibition									
.2		19.6	19.6	-	19.6	19.6	47.8	52.2	62.0	66.3	71.7
.4		27.2	7.9	7.9	22.8	30.7	46.5	53.5	65.8	69.3	72.8
.6		21.4	27.6	33.8	36.6	45.5	57.9	63.4	66.9	69.7	75.9
.8		31.3	33.7	33.7	39.2	52.4	47.0	57.8	78.9	81.3	84.3
.9		19.1	22.8	22.8	29.4	41.9	32.4	55.1	77.2	80.9	83.8
		Sp.Act.(μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Phenthoate					Malathion				
.2	.092	.083	.079	.066	.061	.048	.061	.053	.048	.035	.031
.4	.114	.088	.074	.070	.057	.044	.092	.074	.061	.048	.035
.6	.145	.088	.079	.066	.053	.039	.101	.088	.061	.048	.044
.8	.166	.092	.074	.061	.053	.039	.096	.088	.074	.061	.057
.9	.136	.083	.070	.057	.048	.035	.096	.088	.070	.057	.053
		Per cent Inhibition									
.2		9.8	14.1	28.3	33.7	47.8	33.7	42.4	47.8	62.0	66.3
.4		22.8	35.1	38.6	50.0	61.4	19.3	35.1	46.5	57.9	69.3
.6		39.3	45.5	54.5	63.4	73.1	30.3	39.3	57.9	66.9	69.7
.8		44.6	55.4	63.3	68.1	76.5	42.2	47.0	55.4	63.3	65.7
.9		39.0	48.5	58.1	64.7	74.3	29.4	35.3	48.5	58.1	61.0

Table 21. *In vitro* inhibition of esterases from microsomes of kidney of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.) 50	100	150	200	250	Pesticide (μg a.i.) 50	100	150	200	250
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Methyl parathion					Quinalphos				
.2	.092	.057	.053	.048	.044	.039	.074	.057	.048	.044	.035
.4	.114	.066	.061	.053	.048	.044	.066	.061	.048	.039	.039
.6	.145	.079	.074	.061	.048	.039	.061	.057	.048	.044	.035
.8	.166	.088	.066	.061	.048	.044	.083	.074	.061	.048	.048
.9	.136	.083	.070	.061	.044	.039	.088	.070	.057	.039	.039
					Per cent Inhibition						
.2		38.0	42.4	47.8	52.2	57.6	19.6	38.0	47.8	52.2	62.0
.4		42.1	46.5	53.5	57.9	61.4	42.1	46.5	57.9	65.8	65.8
.6		45.5	49.0	57.9	66.9	73.1	57.9	60.7	66.9	69.7	75.9
.8		47.0	60.2	63.3	71.1	73.5	50.0	55.4	63.3	71.1	71.1
.9		39.0	48.5	55.1	67.6	71.3	35.3	48.5	58.1	71.3	71.3
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Dimethoate					Monocrotophos				
.2	.092	.083	.070	.066	.057	.039	.061	.053	.053	.048	.044
.4	.114	.079	.074	.066	.061	.048	.092	.079	.079	.074	.061
.6	.145	.092	.083	.066	.057	.053	.083	.074	.070	.061	.057
.8	.166	.096	.088	.070	.066	.048	.079	.074	.070	.057	.057
.9	.136	.096	.083	.066	.053	.039	.074	.070	.066	.048	.044
					Per cent Inhibition						
.2		9.8	23.9	28.3	38.0	57.6	33.7	42.4	42.4	47.8	52.2
.4		30.7	35.1	42.1	46.5	57.9	19.3	30.7	30.7	35.1	46.5
.6		36.6	42.8	54.5	60.7	63.4	42.8	49.0	51.7	57.9	60.7
.8		42.2	47.0	57.8	60.2	71.1	52.4	55.4	57.8	65.7	65.7
.9		29.4	39.0	51.5	61.0	71.3	45.6	48.5	51.5	64.7	67.6

concentration >0.8 μM in the absence of pesticides. Similar results were obtained in the presence of Ethion (Figs.28,25, Tables 22,23). With 150-250 μg a.i. concentration of Phenthoate, Methyl parathion and Monocrotophos showed inhibition with substrate concentration >0.8 μM.

4.3.3.4.5 Splenocyte Microsomal Esterases: The microsomal esterases from splenocytes showed inhibition with substrate concentration >0.8 μM in the absence of pesticides. Similar results were obtained in the presence of various concentrations of Ethion, Monocrotophos, Phorate and Methyl parathion whereas spleen did not show such substrate inhibition in the presence of Malathion (Tables 24,25,Figs.29,23). Raina *et al.*(1990) reported maximum inhibition of plasma cholinesterase (87-94%) and serum carboxyl esterase (51-67%) on 28th day in buffalo calves by repeated oral administration of Dichlorvos.

4.3.3.4.6 Abomasal Mucosal and Duodenal Mucosal Esterases

Inhibition of esterases from abomasal- and duodenal mucosal cells (Figs.30-32, Tables 26-29) by different pesticides was relatively low compared to those from liver, kidney, spleen, brain and testes. Malathion was least inhibitory for esterases of abomasal mucosal cells and did not inhibit the duodenal mucosal cell esterases. In fact the activity of duodenal mucosal cell esterases but not those of abomasal mucosal cell esterases increased in the presence of Malathion, Quinalphos and Dimethoate at various concentrations of substrate and pesticides; Ethion and Phorate at low concentration of substrate and pesticides. Sun and Chen (1993) suggested that carboxylesterase serves both as a catalytic

concentration >0.8 μM in the absence of pesticides. Similar results were obtained in the presence of Ethion (Figs.28,25, Tables 22,23). With 150-250 μg a.i. concentration of Phenthoate, Methyl parathion and Monocrotophos showed inhibition with substrate concentration >0.8 μM.

4.3.3.4.5 Splenocyte Microsomal Esterases: The microsomal esterases from splenocytes showed inhibition with substrate concentration >0.8 μM in the absence of pesticides. Similar results were obtained in the presence of various concentrations of Ethion, Monocrotophos, Phorate and Methyl parathion whereas spleen did not show such substrate inhibition in the presence of Malathion (Tables 24,25,Figs.29,23). Raina *et al.*(1990) reported maximum inhibition of plasma cholinesterase (87-94%) and serum carboxyl esterase (51-67%) on 28th day in buffalo calves by repeated oral administration of Dichlorvos.

4.3.3.4.6 Abomasal Mucosal and Duodenal Mucosal Esterases

Inhibition of esterases from abomasal- and duodenal mucosal cells (Figs.30-32, Tables 26-29) by different pesticides was relatively low compared to those from liver, kidney, spleen, brain and testes. Malathion was least inhibitory for esterases of abomasal mucosal cells and did not inhibit the duodenal mucosal cell esterases. In fact the activity of duodenal mucosal cell esterases but not those of abomasal mucosal cell esterases increased in the presence of Malathion, Quinalphos and Dimethoate at various concentrations of substrate and pesticides; Ethion and Phorate at low concentration of substrate and pesticides. Sun and Chen (1993) suggested that carboxylesterase serves both as a catalytic

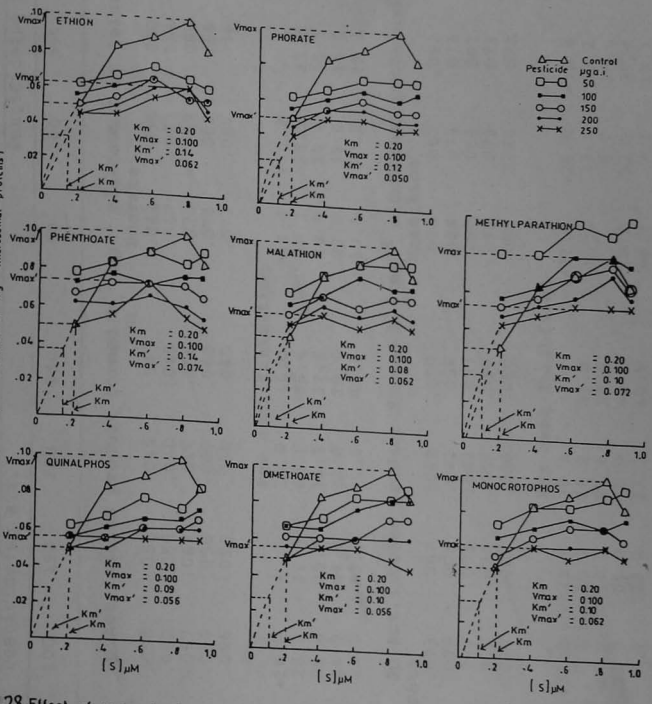


Fig. 28 Effect of concentration of different organophosphorus pesticides (µg a.i.) on the Michaelis–Menten curve of microsomal esterase (S) from Testes of goat (Km and Vmax, control values; and Km' and Vmax' values in the presence of 250 µg a.i.)

Table 22. *In vitro* inhibition of esterases from microsomes of testes of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.) 50	100	150	200	250	Pesticide (μg a.i.) 50	100	150	200	250
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Ethion					**Phorate**				
.2	.050	.062	.056	.050	.045	.045	.062	.056	.050	.045	.039
.4	.084	.067	.062	.056	.050	.045	.067	.062	.056	.056	.050
.6	.090	.073	.067	.067	.062	.056	.073	.067	.062	.056	.050
.8	.100	.067	.056	.056	.062	.062	.073	.062	.056	.050	.045
.9	.084	.062	.056	.056	.050	.045	.073	.067	.056	.050	.045
		Per cent Inhibition									
.2		+19.4	+10.7	-	10.0	10.0	+19.4	+10.7	-	10.0	22.0
.4		20.2	26.2	33.3	40.5	46.4	20.2	26.2	33.3	33.3	40.5
.6		18.9	25.6	25.6	31.1	37.8	18.9	25.6	31.1	37.8	44.4
.8		33.0	44.0	44.0	38.0	38.0	27.0	38.0	44.0	50.0	55.0
.9		26.2	33.3	33.3	40.5	46.4	13.1	20.2	33.3	40.5	46.4
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Phenthoate					**Malathion**				
.2	.050	.078	.073	.067	.062	.050	.073	.067	.062	.056	.056
.4	.084	.084	.078	.073	.062	.056	.084	.073	.073	.067	.062
.6	.090	.090	.073	.073	.067	.073	.090	.084	.067	.062	.056
.8	.100	.084	.078	.073	.062	.056	.090	.078	.073	.067	.062
.9	.084	.090	.078	.067	.056	.050	.090	.078	.073	.062	.056
		Per cent Inhibition									
.2		+35.9	+31.5	+25.4	+19.4	-	+31.5	+25.4	+19.4	+10.7	+10.7
.4		-	7.1	13.1	26.2	33.3	-	13.1	13.1	20.2	20.2
.6		-	18.9	18.9	25.6	18.9	-	6.7	25.6	31.1	37.8
.8		16.0	22.0	27.0	38.0	44.0	10.0	22.0	27.0	33.0	38.0
.9		+6.7	7.1	20.2	33.3	40.5	+6.7	7.1	13.1	26.2	33.3

+, indicates an unexplained increase in activity in presence of the pesticide

Table 23. *In vitro* inhibition of esterases from microsomes of testes of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.) 50	100	150	200	250	Pesticide (μg a.i.) 50	100	150	200	250
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Methyl parathion					**Quinalphos**				
.2	.050	.101	.078	.073	.067	.062	.062	.056	.056	.050	.050
.4	.084	.101	.084	.078	.073	.067	.067	.062	.056	.050	.056
.6	.090	.118	.101	.090	.078	.073	.078	.067	.062	.062	.056
.8	.100	.112	.101	.095	.090	.073	.073	.067	.062	.062	.056
.9	.084	.123	.095	.084	.078	.073	.084	.073	.067	.062	.056
		Per cent Inhibition									
.2		+50.5	+35.9	+31.5	+25.4	+19.4	+19.4	+10.7	+10.7	-	-
.4		+16.8	-	7.1	13.1	20.2	20.2	26.2	33.3	40.5	33.3
.6		+23.7	+10.9	-	13.3	18.9	13.3	25.6	31.1	31.1	37.8
.8		+10.7	-	5.0	10.0	27.0	27.0	33.0	38.0	38.0	44.0
.9		+31.7	+11.6	-	7.1	13.1	-	13.1	20.2	26.2	33.3
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Dimethoate					**Monocrotophos**				
.2	.050	.067	.067	.062	.056	.050	.073	.067	.056	.050	.050
.4	.084	.073	.067	.062	.056	.056	.084	.073	.067	.062	.062
.6	.090	.084	.078	.062	.062	.056	.084	.078	.073	.062	.056
.8	.100	.084	.084	.073	.062	.050	.090	.073	.073	.062	.062
.9	.084	.090	.084	.073	.062	.045	.095	.078	.067	.056	.056
		Per cent Inhibition									
.2		+25.4	+25.4	+19.4	+10.7	-	+31.5	+25.4	+10.7	-	-
.4		13.1	20.2	26.2	33.3	33.3	-	13.1	20.2	26.2	26.2
.6		6.7	13.4	31.1	31.1	37.8	6.7	13.4	18.9	31.1	37.8
.8		16.0	16.0	27.0	38.0	50.0	10.0	27.0	27.0	38.0	38.0
.9		+6.7	-	13.1	26.2	46.4	+11.6	7.1	20.2	33.3	33.3

+, indicates an unexplained increase in activity in presence of the pesticide

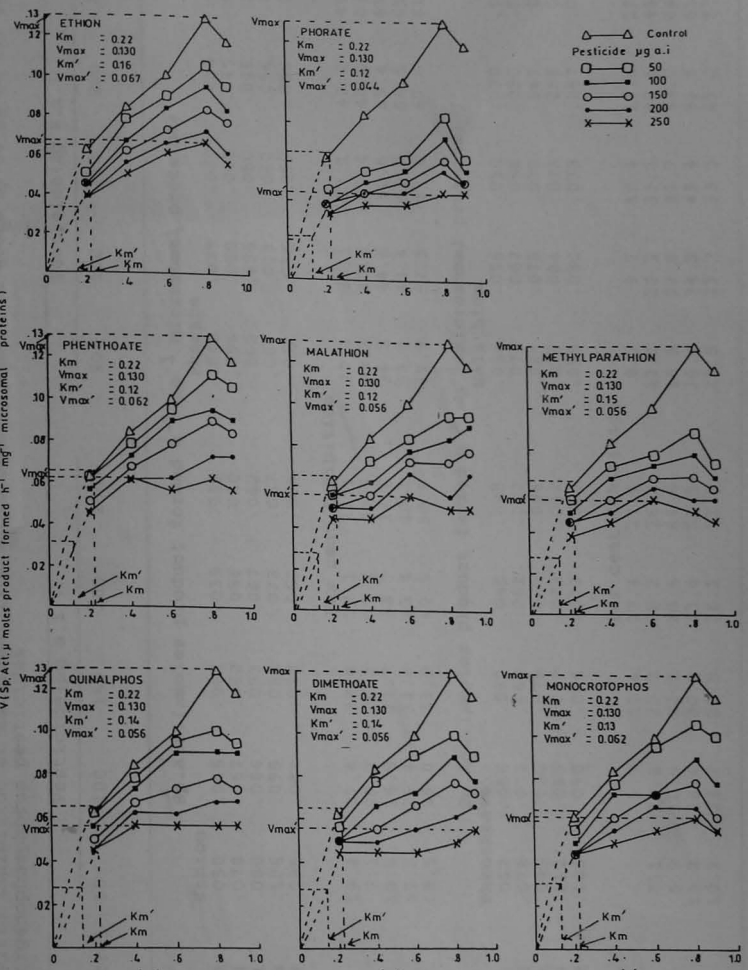


Fig. 29. Effect of concentration of different organophosphorus pesticides (µg a.i.) on the Michaelis – Menten curve of microsomal esterase (s) from Spleen of goat (Km and Vmax, control values; and Km' and Vmax', values in the presence of 250 µg a.i.)

Table 24. *In vitro* inhibition of esterases from microsomes of spleen of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.) 50	100	150	200	250	Pesticide (μg a.i.) 50	100	150	200	250
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Ethion					Phorate				
.2	.062	.050	.045	.045	.039	.039	.045	.039	.039	.034	.034
.4	.084	.078	.067	.062	.056	.050	.056	.050	.045	.045	.039
.6	.101	.090	.084	.073	.067	.062	.062	.056	.050	.045	.039
.8	.129	.106	.095	.084	.073	.067	.084	.073.	.062	.056	.045
.9	.118	.095	.084	.078	.062	.056	.062	.056	.050	.050	.045
		Per cent Inhibition									
.2		19.4	27.4	27.4	37.1	37.1	27.4	37.1	37.1	45.2	45.2
.4		7.1	20.2	26.2	33.3	40.5	33.3	40.5	46.4	46.4	53.6
.6		10.9	16.8	27.7	33.7	38.6	38.6	44.6	50.5	55.4	61.4
.8		17.8	26.4	34.9	43.4	48.1	34.9	43.4	51.9	56.6	65.1
.9		19.5	28.8	33.9	47.5	52.5	47.5	52.5	57.6	57.6	61.9
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Phenthoate					Malathion				
.2	.062	.062	.056	.050	.045	.045	.056	.056	.050	.050	.045
.4	.084	.078	.073	.067	.062	.062	.073	.062	.056	.050	.045
.6	.101	.095	.090	.078	.062	.056	.084	.078	.073	.067	.056
.8	.129	.112	.095	.090	.073	.062	.095	.084	.073	.056	.050
.9	.118	.106	.090	.084	.073	.056	.095	.090	.078	.067	.050
		Per cent Inhibition									
.2		-	9.7	19.4	27.4	27.4	9.7	9.7	19.4	19.4	27.4
.4		7.1	13.1	20.2	26.2	26.2	13.1	26.2	33.3	40.5	46.4
.6		5.9	10.9	22.8	38.6	44.6	16.8	22.8	27.7	33.7	44.6
.8		13.2	26.4	30.2	43.4	51.9	26.4	34.9	43.4	56.6	61.2
.9		10.2	23.7	28.8	38.1	52.5	19.5	23.7	33.9	43.2	57.6

Table 25. *In vitro* inhibition of esterases from microsomes of spleen of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.)					Pesticide (μg a.i.)				
		50	100	150	200	250	50	100	150	200	250
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Methyl parathion					Quinalphos				
.2	.062	.056	.050	.045	.045	.039	.062	.056	.050	.045	.045
.4	.084	.073	.067	.056	.050	.045	.078	.073	.067	.062	.056
.6	.101	.078	.073	.067	.062	.056	.095	.090	.073	.062	.056
.8	.129	.090	.078	.067	.056	.050	.101	.090	.078	.067	.056
.9	.118	.073	.067	.062	.056	.045	.095	.090	.073	.067	.056
		Per cent Inhibition									
.2		9.7	19.4	27.4	27.4	37.1	-	9.7	19.4	27.4	27.4
.4		13.1	20.2	33.3	40.5	46.4	7.1	13.1	20.2	26.2	33.3
.6		22.8	27.7	33.7	38.2	44.6	5.9	10.9	27.7	38.2	44.6
.8		30.2	39.5	48.1	56.6	61.2	21.7	30.2	39.5	48.1	56.6
.9		38.1	43.2	47.5	52.5	61.9	19.5	23.7	38.1	43.2	52.5
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Dimethoate					Monocrotophos				
.2	.062	.056	.050	.050	.050	.045	.056	.050	.045	.045	.045
.4	.084	.078	.067	.056	.050	.045	.078	.073	.062	.056	.050
.6	.101	.090	.073	.067	.056	.045	.095	.073	.073	.067	.056
.8	.129	.101	.090	.078	.062	.050	.106	.090	.078	.067	.062
.9	.118	.090	.078	.073	.067	.056	.101	.078	.062	.056	.056
		Per cent Inhibition									
.2		9.7	19.4	19.4	19.4	27.4	9.7	19.4	27.4	27.4	27.4
.4		7.1	20.2	33.3	40.5	46.4	7.1	13.1	26.2	33.3	40.5
.6		10.9	27.7	33.7	44.6	55.4	5.9	27.7	27.7	33.7	44.6
.8		21.7	30.2	39.5	51.9	61.2	17.8	30.2	39.5	48.1	51.9
.9		23.7	33.9	38.1	43.2	52.5	14.4	33.9	47.5	52.5	52.5

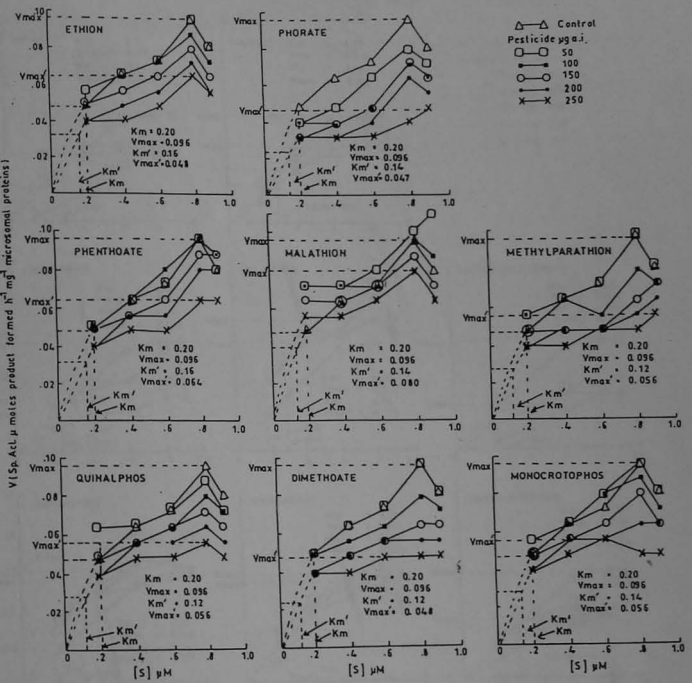


Fig. 30. Effect of concentration of different organophosphorus pesticides (µg a.i.) on the Michaelis-Menten curve of esterase (s) from Abomasal mucosa of goat (Km and Vmax, control values; and Km' and Vmax', values in the presence of 250 µg a.i.)

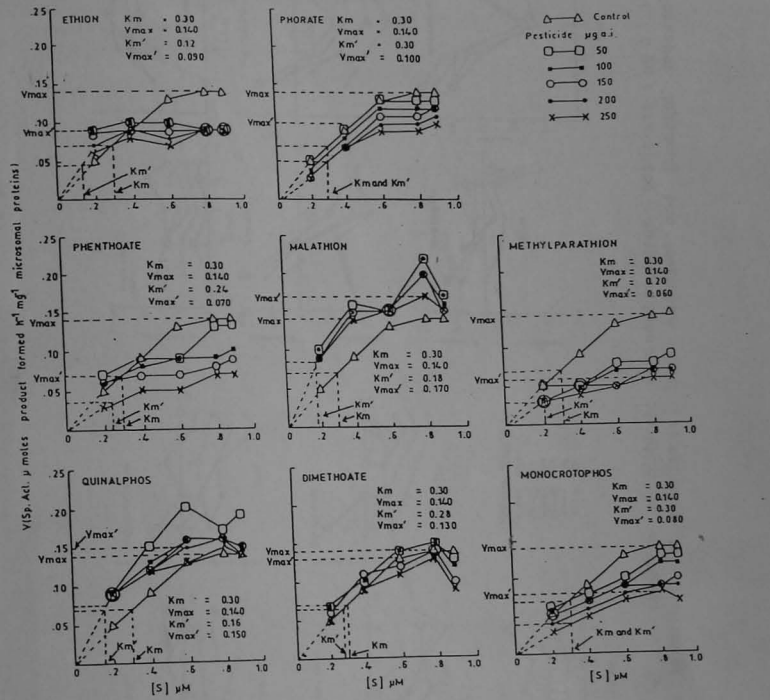


Fig. 31. Effect of concentration of different organophosphorus pesticides (µg a.i.) on the Michaelis – Menten curve of esterase (s) from Duodenal mucosa of goat (Km and Vmax, control values; and Km' and Vmax', values in the presence of 250 µg a.i.)

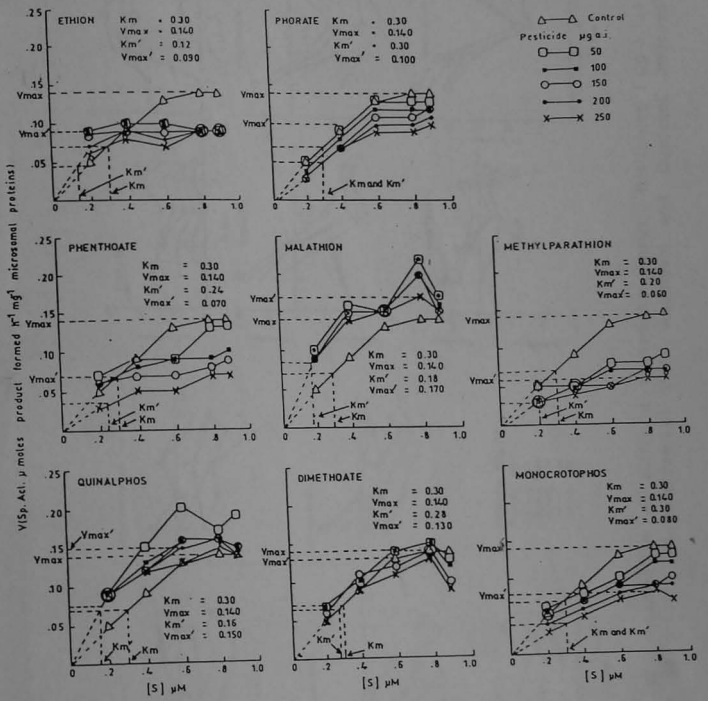


Fig. 31. Effect of concentration of different organophosphorus pesticides (µg a.i.) on the Michaelis – Menten curve of esterase (s) from Duodenal mucosa of goat (Km and Vmax, control values; and Km' and Vmax', values in the presence of 250 µg a.i.)

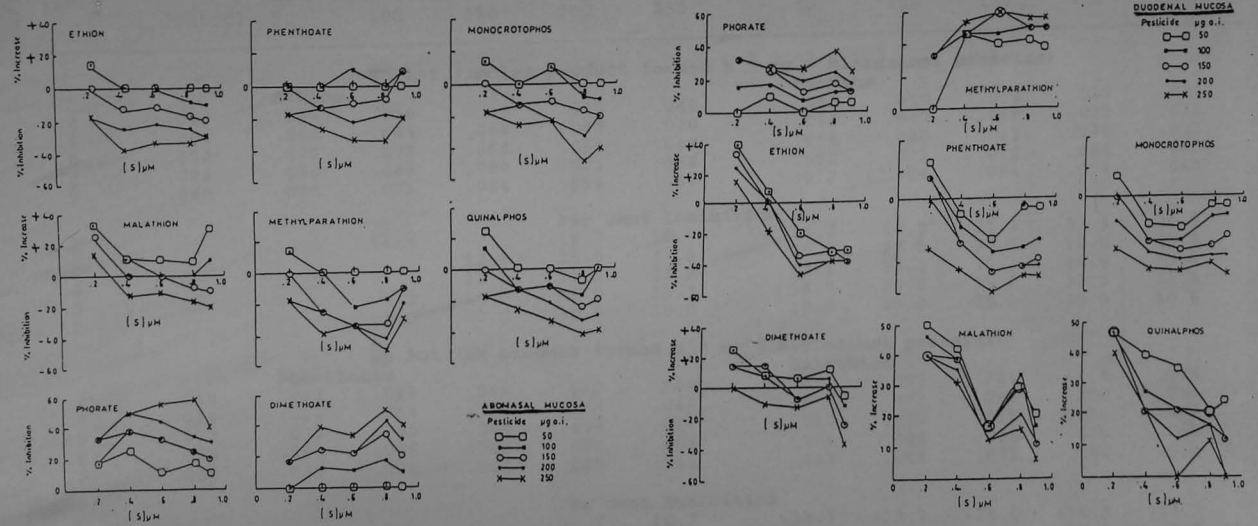


Fig. 32. Effect of concentration of different organophosphorus pesticides (µg a.i.) on Percent change in activity of esterase (s) from Abomasal and Duodenal mucosal cells.

Table 26. *In vitro* inhibition of esterases from abomasal mucosal cells of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.) 50	100	150	200	250	Pesticide (μg a.i.) 50	100	150	200	250
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Ethion					**Phorate**				
.2	.048	.056	.056	.048	.040	.040	.040	.040	.032	.032	.032
.4	.064	.064	.064	.056	.048	.040	.048	.040	.040	.032	.032
.6	.072	.072	.072	.064	.056	.048	.064	.048	.048	.040	.032
.8	.096	.096	.088	.080	.072	.064	.080	.072	.072	.064	.040
.9	.080	.080	.072	.064	.056	.056	.072	.064	.064	.056	.040
		Per cent Inhibition									
.2		+14.3	+14.3	-	16.7	16.7	16.7	16.7	33.3	33.3	33.3
.4		-	-	12.5	25.0	37.5	25.0	37.5	37.5	50.0	50.0
.6		-	-	11.1	22.2	33.3	11.1	33.3	33.3	44.4	55.5
.8		-	8.3	16.7	25.0	33.3	16.7	25.0	25.0	33.3	58.3
.9		-	10.0	20.0	30.0	30.0	10.0	20.0	20.0	30.0	40.0
		Sp.Act. (μM product formed h^{-1} mg^{-1} microsomal proteins)									
		Phenthoate					**Malathion**				
.2	.048	.048	.048	.048	.040	.040	.072	.072	.064	.056	.056
.4	.064	.064	.064	.056	.056	.048	.072	.072	.064	.056	.056
.6	.072	.072	.080	.064	.056	.048	.080	.072	.072	.064	.064
.8	.096	.096	.096	.088	.080	.064	.104	.096	.088	.080	.080
.9	.080	.080	.088	.088	.080	.064	.112	.088	.072	.064	.064
		Per cent Inhibition									
.2		-	-	-	16.7	16.7	+33.3	+33.3	+25.0	+14.3	+14.3
.4		-	-	12.5	12.5	25.0	+11.1	+11.1	-	12.5	12.5
.6		-	+10.0	11.1	22.2	33.3	+10.0	-	-	11.1	11.1
.8		-	-	8.3	16.7	33.3	+7.7	-	8.3	16.7	16.7
.9		-	+9.1	+9.1	-	20.0	+28.6	+9.1	10.0	20.0	20.0

+, indicates an unexplained increase in activity in presence of the pesticide

Table 27. *In vitro* inhibition of esterases from abomasal mucosal cells of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.) 50	100	150	200	250	Pesticide (μg a.i.) 50	100	150	200	250
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Methyl parathion					**Quinalphos**				
.2	.048	.056	.056	.048	.040	.040	.064	.056	.048	.040	.040
.4	.064	.064	.064	.048	.048	.040	.064	.056	.056	.056	.048
.6	.072	.072	.056	.048	.048	.048	.072	.064	.064	.056	.048
.8	.096	.096	.080	.064	.056	.048	.088	.080	.072	.064	.056
.9	.080	.080	.072	.072	.064	.056	.072	.072	.064	.056	.048
					Per cent Inhibition						
.2		+14.3	+14.3	-	16.7	16.7	+25.0	+14.3	-	16.7	16.7
.4		-	-	25.0	25.0	37.5	-	12.5	12.5	12.5	25.0
.6		-	22.2	33.3	33.3	33.3	-	11.1	11.1	22.2	33.3
.8		-	16.7	33.3	41.7	50.0	8.3	16.7	25.0	33.3	41.7
.9		-	10.0	10.0	20.0	30.0	-	-	20.0	30.0	40.0
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Dimethoate					**Monocrotophos**				
.2	.048	.048	.048	.040	.040	.040	.056	.048	.048	.040	.040
.4	.064	.064	.056	.048	.048	.040	.064	.064	.056	.056	.048
.6	.072	.072	.064	.056	.056	.048	.080	.080	.064	.056	.056
.8	.096	.096	.080	.064	.056	.048	.096	.088	.080	.064	.048
.9	.080	.080	.072	.064	.056	.048	.080	.072	.064	.064	.048
					Per cent Inhibition						
.2		-	-	16.7	16.7	16.7	+14.3	-	-	16.7	16.7
.4		-	12.5	25.0	25.0	37.5	-	-	12.5	12.5	25.0
.6		-	11.1	22.2	22.2	33.3	+10.0	+10.0	11.1	22.2	22.2
.8		-	16.7	33.3	41.7	50.0	-	8.3	16.7	33.3	50.0
.9		-	10.0	20.0	30.0	40.0	-	10.0	20.0	20.0	40.0

+, indicates an unexplained increase in activity in presence of the pesticide

Table 28. *In vitro* inhibition of esterases from duodenal mucosal cells of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.) 50	100	150	200	250	Pesticide (μg a.i.) 50	100	150	200	250
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Ethion					**Phorate**				
.2	.051	.085	.085	.077	.068	.060	.051	.043	.034	.034	.034
.4	.094	.102	.102	.094	.094	.077	.085	.077	.068	.068	.068
.6	.128	.102	.102	.085	.077	.068	.128	.119	.111	.102	.094
.8	.136	.094	.094	.094	.085	.085	.128	.119	.111	.102	.085
.9	.136	.094	.094	.085	.085	.085	.128	.119	.119	.111	.102
		Per cent Inhibition									
.2		+40.0	+40.0	+33.8	+25.0	+15.0	-	15.7	33.3	33.3	33.3
.4		+ 7.8	+ 7.8	-	-	18.1	9.6	18.1	27.7	27.7	27.7
.6		20.3	20.3	33.6	39.8	46.9	-	7.0	13.3	20.3	26.6
.8		30.9	30.9	30.9	37.5	37.5	5.9	12.5	18.4	25.0	37.5
.9		30.9	30.9	37.5	37.5	37.5	5.9	12.5	12.5	18.4	25.0
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Phenthoate					**Malathion**				
.2	.051	.068	.060	.060	.051	.034	.102	.094	.085	.085	.085
.4	.094	.085	.077	.068	.068	.051	.162	.153	.153	.145	.136
.6	.128	.094	.085	.068	.068	.051	.153	.153	.153	.145	.145
.8	.136	.128	.094	.077	.077	.068	.221	.204	.196	.170	.162
.9	.136	.128	.102	.085	.077	.068	.170	.162	.153	.153	.145
		Per cent Inhibition									
.2		+25.0	+15.0	+15.0	-	33.3	+50.0	+45.7	+40.0	+40.0	+40.0
.4		9.6	18.1	27.7	27.7	45.7	+42.0	+38.6	+38.6	+35.2	+30.9
.6		26.6	33.6	46.9	46.9	60.2	+16.3	+16.3	+16.3	+11.7	+11.7
.8		5.9	30.9	43.4	43.4	50.0	+38.5	+33.3	+30.6	+20.0	+16.0
.9		5.9	25.0	37.5	43.4	50.0	+20.0	+16.0	+11.1	+11.1	+ 6.2

+, indicates an unexplained increase in activity in presence of the pesticide

Table 29. *In vitro* inhibition of esterases from duodenal mucosal cells of goats by 50 μg to 250 μg a.i. of organophosphorus pesticides

Substrate (μM)	Control	Pesticide (μg a.i.)					Pesticide (μg a.i.)				
		50	100	150	200	250	50	100	150	200	250
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Methyl parathion					Quinalphos				
.2	.051	.051	.034	.034	.034	.034	.094	.094	.094	.094	.085
.4	.094	.051	.051	.051	.043	.043	.153	.128	.119	.119	.119
.6	.128	.077	.068	.051	.051	.051	.196	.162	.162	.145	.128
.8	.136	.077	.068	.068	.068	.060	.170	.162	.170	.162	.153
.9	.136	.085	.068	.068	.068	.060	.187	.153	.153	.153	.136
		Per cent Inhibition									
.2		-	33.3	33.3	33.3	33.3	+45.7	+45.7	+45.7	+45.7	+40.0
.4		45.7	45.7	45.7	54.3	54.3	+38.6	+26.7	+21.0	+21.0	+21.0
.6		39.8	46.9	60.2	60.2	60.2	+34.7	+21.0	+21.0	+11.7	-
.8		43.4	50.0	50.0	50.0	55.9	+20.0	+16.0	+20.0	+16.0	+11.1
.9		37.5	50.0	50.0	50.0	55.9	+27.3	+11.1	+11.1	-	-
		Sp.Act. (μmoles product formed h^{-1} mg^{-1} microsomal proteins)									
		Dimethoate					Monocrotophos				
.2	.051	.068	.068	.060	.060	.051	.060	.051	.051	.043	.034
.4	.094	.102	.102	.111	.102	.085	.077	.068	.068	.060	.051
.6	.128	.136	.136	.119	.119	.111	.102	.094	.085	.077	.068
.8	.136	.153	.145	.136	.136	.128	.128	.119	.094	.085	.077
.9	.136	.128	.119	.102	.102	.085	.128	.119	.102	.085	.068
		Per cent Inhibition									
.2		+25.0	+25.0	+15.0	+15.0	-	+15.0	-	-	15.7	33.3
.4		+7.8	+7.8	+15.3	+7.8	9.6	18.1	27.7	27.7	36.2	45.7
.6		+5.9	+5.9	7.0	7.0	13.3	20.3	26.6	33.6	39.8	46.9
.8		+11.1	+6.2	-	-	5.9	5.9	12.5	30.9	37.5	43.4
.9		5.9	12.5	25.0	25.0	37.5	5.9	12.5	25.0	37.5	50.0

+, indicates an unexplained increase in activity in presence of the pesticide

protein for the hydrolysis of some insecticides (e.g. Malathion and Transpermethrin) and a binding protein for the oxons of the several organophosphorus compounds, some carbamates and pyrethroids.

As in the control, the substrate concentration >0.8 μM produced substrate inhibition of abomasal esterases in the presence of various concentrations of Ethion, Quinalphos, Phorate (except 250 μg a.i.) and Malathion (except 50 μg a.i.). Phorate inhibited abomasal esterases and duodenal esterases did not show substrate inhibition as was observed with esterases of microsomal preparations from other tissues. In general, Methyl parathion, Phenthoate and Monocrotophos maximally inhibited the duodenal mucosal cell esterases. It was concluded that the duodenal and abomasal mucosal cell esterases are predominantly hydrolytic in nature and are inhibited only to a limited extent by increasing concentration of all the pesticides except Quinalphos and Malathion. The observed increase in hydrolysis of substrate, in the presence of various concentrations of Malathion and Quinalphos was difficult to explain and needs more detailed investigations. It was further concluded that organophosphorus pesticides entering the digestive tract are sequestered by the proteins/enzymes of digestive tract and the proportion of pesticides which may be absorbed can also be sequestered and degraded by the esterases of the digestive tract mucosal cells before entering other tissues. Main and Braid (1962) reported that Malathion with intact ester group has LD_{50} of 20 mg kg^{-1} b.w. for male rats whereas its oxidative desulfuration decreases the LD_{50} to 158 mg kg^{-1} b.w. for male

rats suggesting that the intactness of carboxylester linkage is important for the toxicity of Malathion. *In vivo* LD_{50} of Malathion for male rats is 2800 mg kg^{-1} b.w. (Martin, 1968).

The comparative structures and toxicity studies with these eight pesticides showed that Phosphorothioates and Phosphates with aliphatic and aromatic groups mainly affect the organisation and functions of microsomes from brain and kidneys. The presence of aromatic group in Phosphorothioates also produces such effects in spleen and/or testes. Dithiophosphates with thioether or carbamide linkage in the side chain affect testes also. Dithiophosphates with aromatic ring and single carboxyl ester linkage in the side chain and Phosphates with methylcarbamide linkage in the side chain preferentially affect the brain and kidneys. Thiophosphates with Nitrophenyl or heterocyclic aromatic ring in the side chain also affect testes and spleen. Dithiophosphates with two carboxylester linkages have stronger effects on kidneys and spleen.

From these studies the following conclusions were drawn:

- The pesticides had 4 to 8 phosphorus containing components in different proportions and showed positive reaction with Lowry reagents.
- Complexes of Methyl parathion and Quinalphos with Lowry reagents can be estimated at 410 nm and 380 nm, respectively; Dimethoate, Monocrotophos and Malathion at 660 nm; and Ethion, Phorate and Phenthoate at 520 nm or 660 nm.

- The presence of Quinalphos (Rf value (0.93), Methyl parathion (Rf value 0.91) and Dimethoate (Rf value 0.93) was detected by TLC at 624 ppb, 938 ppb and 521 ppb levels, respectively.
- The binding to and inhibition of pepsin by the eight organophosphorus pesticides was higher than that of trypsin.
- The binding of these pesticides to microsomes from brain was higher, compared to those from testes, kidneys, spleen and liver.
- The binding of organophosphorus pesticides to microsomes from spleen, brain and kidneys was at lipophillic site away from heme and directly to heme in microsomes from testes and liver.
- The Absolute Absorption Spectra of microsomal preparations from liver, spleen, brain, kidneys and testes of goats showed hyperchromic effect with all the pesticides except hypochromic effect of Phenthoate and Malathion on microsomes of liver. The results suggest that these pesticides disorganise the microsomes from different tissues.
- Kinetic studies of microsomal esterases indicated the presence of at least two esterases in abomasal-, duodenal mucosal cells and in microsomes from brain, spleen, testes, kidneys and liver.
- Substrate concentration > 0.8 μM were inhibitory for esterases from spleen, brain, testes, liver and kidneys of goats.

- Increasing concentration of pesticides inhibited the microsomal esterases from these tissues of goats.
- The decrease in the Km and Vmax values of esterases in the presence of pesticides indicated modification of affinity of the substrate for the esterases.
- The comparative structures and toxicity studies with these eight pesticides showed that Phosphorothioates and Phosphates with aliphatic and aromatic groups mainly affect the organisation and functions of microsomes from brain and kidneys. Dithiophosphates with thioether or carbamide linkage in the side chain affect testes also. The presence of aromatic group in Phosphorothioates also produces such effects in spleen and/or testes.

CHAPTER V

SUMMARY

Excessive and indiscriminate use of chemicals such as pesticides and fertilizers to increase agricultural production, modifies the ecosystem. The toxicity due to chronic exposure to residues of such chemicals especially organophosphorus pesticides has been investigated to a limited extent. Using tissues of goats an *in vitro* study was, therefore, carried out with Ethion, Phorate, Phenthoate, Malathion, Methyl parathion, Quinalphos, Dimethoate and Monocrotophos with the following objectives: to fractionate, characterize and estimate the constituents of commercial organophosphorus pesticides; to understand the biochemical basis of toxicity of organophosphorus pesticides from Absolute Absorption Spectra of pesticides, microsomes and their complexes; binding of pesticides to biomolecules; binding and *in vitro* inhibition of digestive enzymes and microsomal esterases of different tissues of goats.

TLC of eight commercial organophosphorus pesticides indicated the presence of four to eight phosphorus containing components. Component of Methyl parathion (Rf 0.40), Quinalphos (Rf 0.75) respectively showed mustard yellow and blue-green diffused colour. Three components (Rf 0.75, 0.51 and 0.36) of Dimethoate showed pink colour with iodine vapour, which

gradually changed to greyish brown. The components of Methyl parathion (Rf 0.81) and Quinalphos (Rf 0.93), respectively remained yellow and blue green instead of blue after reaction with Lowry reagents. The detection limits of components of Quinalphos (Rf 0.93), Methyl parathion (Rf 0.81) and Dimethoate (Rf 0.93) were 624 ppb, 938 ppb and 521 ppb, respectively after visualization with iodine vapour and Lowry reagents. From the Absorption Spectra of complexes of organophosphorus pesticides with Lowry reagents, it was concluded that Dimethoate, Monocrotophos and Malathion may be estimated at 660 nm; Methyl parathion and Quinalphos at 410 nm and 380 nm, respectively; and Ethion, Phenthoate and Phorate both at 660 nm or 520 nm.

The binding of the eight organophosphorus pesticides to BSA and casein was higher than that to carbohydrates; their binding to trypsin and pepsin increased with increasing concentration of pesticides (50 µg to 250 µg a.i.). The binding of pesticides to trypsin was in the following decreasing order: Malathion < Quinalphos < Ethion (Phorate) < Phenthoate (Methyl parathion) < Dimethoate and < Monocrotophos. The decreasing order of binding of pesticides to pepsin was: Phorate < Methyl parathion < Dimethoate < Phenthoate (Monocrotophos) < Quinalphos and < Ethion (Malathion). The inhibition of pepsin by all the eight organophosphorus pesticides was higher, compared to that of trypsin. The highest inhibition of trypsin (80%) and pepsin (86.5%) was achieved with 250 µg a.i. of Phorate and Phenthoate, respectively. Inhibition of trypsin in decreasing order was: Phorate < Quinalphos < Dimethoate < Ethion < Methyl parathion < Malathion < Phenthoate and <

Monocrotophos; and that of pepsin was: Phenthoate < Quinalphos < Phorate < Ethion < Dimethoate < Methyl parathion < Malathion and < Monocrotophos. Inhibition of pepsin appears to be higher than that of trypsin. The binding of organophosphorus pesticides was highest to the microsomes from brain of goats. The binding of various pesticides to microsomes was: 100% for Phorate and Dimethoate to microsomes from brain; 89.4% for Quinalphos and 88.3% for Dimethoate to microsomes from kidneys; 93.3% for Phorate to microsomes from testicular cells; 77.1% for Quinalphos and 76.7% for Phenthoate to microsomes from liver and 78.8% for Quinalphos to microsomes from splenocytes.

The Absolute Absorption Spectra of microsomal preparations from liver, kidney, spleen, brain and testes of goats showed hyperchromic effect with all the pesticides. However, with Phenthoate and Malathion the effect was hypochromic on Spectra of microsomes from liver. The Absorption Difference Spectra indicated that the binding of organophosphorus pesticides (except Methyl parathion) to the microsomes from splenocytes was only at lipophillic sites - (Type I) away from heme in microsomes. Binding of all the pesticides except Methyl parathion and Phenthoate appears to be to heme - (Type II and/or Reverse Type I) component of microsomes from testicular cells. Same appears to be the case with microsomes from the liver except with Quinalphos and Dimethoate, brain (only with Ethion and Phorate) and kidney cells (only with Methyl parathion). Since these pesticides apparently bind and modify the heme of Cyt.P-450 in microsomes of liver and testicular cells, so functions of liver and testes

may be adversely affected. In absence of pesticides the calculated functional Cyt.P-450 content (change in AU mg^{-1} microsomal proteins) of goats was 4.44 for testes, 4.27 for brain, 3.74 for kidney, 3.20 for liver and 1.58 for spleen, respectively. The calculated functional Cyt.P-450 content of microsomes from liver and testes was decreased by all the pesticides except Methyl parathion; and that of microsomes from kidneys, spleen and brain was increased except by Monocrotophos and Phorate; Phenthoate; and Phorate and Ethion respectively.

The esterases in duodenal- and abomasal mucosal cells and microsomes from brain showed maximum activities at pH 8.0-8.5 and pH 8.0, respectively. The esterases of microsomes from testes, liver, kidneys and spleen showed maximum activity at pH 7.4 and in decreasing order. The optimum temperature for esterases of abomasal- and duodenal mucosal cells and of microsomes from liver, kidney, testes, brain was 37°C whereas those from splenocytes was 45°C. The activation energy (calculated form Arrhenius plot) of esterases in microsomes from spleen, kidneys, testes, liver and brain was 6072, 6831, 7970, 11021, 14801 and Ea of esterases from abomasal- and duodenal mucosal cells was 3038 and 4554 cal $mole^{-1}$, respectively.

The low and high Km values of esterases in the microsomes from kidneys and testes suggested the presence of enzyme having high and low affinity for the substrate. The esterases in duodenal mucosal cells and microsomes from brain also appear to have low affinity for the substrate, but the Vmax was highest for esterases from brain. At least two

esterases appear to be present in various abomasal and duodenal mucosal cells and microsomes from liver, kidney, brain, testes and spleen. The Lineweaver-Burk plot suggested that the esterases in abomasal-, duodenal mucosal cells and microsomes from liver, kidney, brain, spleen and testes showed substrate inhibition at substrate concentration > 0.8 μM. The inhibition of esterases in microsomes from liver, kidney, spleen and brain by all the eight pesticides was concentration dependent. This trend was partially reversed by increasing the substrate concentration. Inhibition of esterases in abomasal- and duodenal mucosal cells of goats by different pesticides was relatively low compared to that in microsomes from liver, kidney, spleen, brain and testes of goats. From the binding and enzyme inhibition studies it appears that proteins and hydrolytic enzymes sequester the pesticides and add to the detoxification of pesticides. The comparative structures and toxicity studies with these eight pesticides showed that Phosphorothioates and Phosphates with aliphatic and aromatic groups mainly affect the organisation and functions of microsomes from brain and kidneys. Dithiophosphates with thioether or carbamide linkage in the side chain affect testes also. The presence of aromatic group in Phosphorothioate also produces such effects in spleen and/or testes.

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